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ARTICLE

Structural variations on *Salmonella* biofilm by exposition to river water

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ABSTRACT

Biofilm formation, as adapting strategies, is the result of stressful conditions that *Salmonella* faces in hostile environments like surface water. We evaluated river water effect on *Salmonella* biofilm formation ability in terms of physical, morphological characteristics and chemical composition. A new morphotype SPAM (soft, pink and mucoid) was detected in Oranienburg strains S-76 and S-347 (environmental and clinical isolate). Oranienburg serotypes showed very marked behavior in adherence, pellicle liquid-air and resistance, being Oranienburg S-76 the strongest biofilm producer. All strains when exposed to river water presented an overlapping mucoid layer in the morphotype and increased their motility except Oranienburg S-347. The most motile was Typhimurium (control) and the least Infantis S-304 (clinical isolate). Mannose, glucose, galactose and ribose were the main biofilm sugar components; type and concentration of sugar suggest a morphotype/serotype dependent pattern. Strong morphotypes expressed in this study may be an effective protective strategy for *Salmonella* in hostile environments.

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KEYWORDS
Salmonella; biofilm composition; morphotype; river water exposition; non-host environment

Introduction

Salmonella is a major public health concern and a significant cause of morbidity and mortality worldwide (WHO 2018). This bacterium can widespread in natural settings, being water the main vehicle of transmission (Levantesi et al. 2012; Liu et al. 2018; WHO 2018). Water is a harsh environment for most vegetative bacteria; thus, the deployment of survival mechanisms is needed, including the formation of a dynamic microbial cell community known as biofilms, which is the main microbiological protection strategy. Biofilms not only provide protection to *Salmonella* against desiccation and disinfection conditions, but deal in driving the bacterium to a long-term persistence in the environment (Anriany et al. 2001; Vestby et al. 2009). The best protective strategy displayed by *Salmonella* in hostile environments is undoubtedly biofilms as stated by Gaertner et al. (2011) and Sha et al. (2011). Nevertheless, it has not yet been established whether the conditions of water exposure alter the biofilm capacity and the persistence of *Salmonella* in natural settings. Biofilms are structures with rough and elevated three-dimensional characteristics that are irreversibly associated to biotic and

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abiotic surfaces, enclosed in a matrix of self-produced extracellular polymers, including a perfect channeling architecture allowing the influx of nutrients and oxygen and the efflux of waste substances (Hall-Stoodley et al. 2004; Steenackers et al. 2012; Singh et al. 2017; Medrano-Félix et al. 2018). Biofilm characteristics and composition may vary among bacterial genera. In the case of *Salmonella* exopolysaccharides (EPS) as cellulose, colanic acid and O-antigen capsule, proteins such as curli fimbriae and BapA are the main constituents; nonetheless environmental conditions and substratum facing bacteria influence quantity of biofilm components and morphology (Solano et al. 2002; Steenackers et al. 2012). The first approach to study the biofilms characteristics and composition is at a laboratory-level, involving adhesion to surfaces, pellicle liquid-air, and morphotype assays that indicate their response to diverse stimuli (White and Surette 2006; Steenackers et al. 2012). To date, curli fimbriae and cellulose structures reflected in the strong aggregative RDAR phenotype (red, dry, and rough) is mainly observed in *Salmonella* biofilms. RDAR morphotype is hypothesized to represent a critical state in the transmission of *Salmonella* between hosts (Steenackers et al. 2012; Milanov et al. 2015; Singh et al. 2017). Other *Salmonella* morphotypes have been reported forming weak biofilms; i.e., PDAR (pink, dry and rough) and BDAR (brown, dry and rough) which express cellulose and curli, respectively; while, SAW (soft and white) express neither curli nor cellulose; SBAM (soft, brown and mucoid) and BAS (brown and soft) morphotype generate an overproduction of capsular polysaccharides (Römling et al. 2003; Malcova et al. 2008; Ramachandran et al. 2016).

Despite the diversity of studies about *Salmonella* biofilm formation in abiotic surfaces, plant surfaces or animal epithelial cells (Solano et al. 2002; White and Surette 2006; Steenackers et al. 2012); there is a constraint on whether biofilm formation favors the persistence of *Salmonella* in a non-host environment such as water (Gaertner et al. 2011; Sha et al. 2011; Liu et al. 2018; López Cuevas et al. 2009). In this regard, studies comparing clinical and environmental isolates are scarce (Römling et al. 2003; Ramachandran et al. 2016; MacKenzie et al. 2017; Medrano-Félix et al. 2018). Therefore, more studies are needed to increase the basic knowledge about biofilm composition under water conditions.

This study evaluated biofilm-formation ability and the characteristics in *Salmonella* Oranienburg, Saintpaul and Infantis, to better understand their prevalence in aquatic environments. In this regard, the knowledge about biofilm-forming process, characteristics and composition of biofilms developed by *Salmonella* exposed to unfavorable environmental conditions, such as river water can contribute to the better comprehension about the behavior of *Salmonella* in the environment.

Materials and methods

Reactivation and preparation of Salmonella strains

Environmental and clinical *Salmonell*a strains were included in the study. From river water, serotypes Saintpaul S-70, Oranienburg S-76 and Infantis S-285, and from human stool Infantis S-304 and Oranienburg S-347 all were subjected to analysis. *Salmonella* Typhimurium ATCC 14028 was used as control. The original codes assigned to the strains of environmental isolates were abbreviated as 'Env' (Saintpaul Env S-70, Oranienburg Env S-76, Infantis Env S-285) and the clinical isolates as 'Cli' (Infantis Cli S-304, Oranienburg Cli S-347) and were used throughout the text when it was necessary to refer to the strains individually. All strains were kindly provided by the National Laboratory for Research in Food Safety (LANIIA, for its acronym in Spanish) and were reactivated in selective medium Xylose Lysine Deoxycholate (XLD) (BD Bioxon, México) and incubated at 37°C for 24 h. Once reactivated, strains were cultured in 50 mL of Luria Bertani (LB) broth and incubated at 37°C during 18 h under aerobic conditions (Shell-lab, Cornelius OR, USA). After incubation, bacterial cells were pelleted by centrifugation (Thermo Scientific, MA, USA) at 10,000 *g* for 10 min at 4°C, the supernatant was discarded, and the cell pellet was washed twice to minimize cellular debris. The resuspension and the serial dilutions were performed using

phosphate buffered solution (PBS) 0.01 M to obtain a cell suspension OD_{600} of 1 (ca. 10^8 $CFUmL^{-1}$). Bacterial titer was confirmed by spread plate on XLD agar using decimal dilutions.

Biofilms of *Salmonella* strains were subjected to analysis after being formed when exposed to two scenarios: 1) laboratory conditions and 2) exposition to river water.

Survival chambers and exposition to river water

For *Salmonella* exposition to river water sterile survival chambers were used as a microcosm for each strain. Bacterial suspension OD_{600} of 1 was inoculated in each chamber (per triplicate) according to Medrano-Félix et al. (2017) with brief modifications. Exposition to river water was performed at Humaya river (24º 49′ 46.4″N, 107º 24′01. 1″W), chambers were exposed for 96 hours to river water to expose bacteria to this non-host environment. After exposure time, survival chambers were collected and aseptically transported to the laboratory for biofilm analysis. Prior to analysis, external parts of each chamber were disinfected using ethanol (70%) to avoid the contamination of the inoculum, which was transferred to 50 mL conical tubes for further analysis. To ensure the absence of contamination after exposition to river water, an aliquot of each recovered inoculum was plated on XLD agar to observe the solely presence of typical colonies of *Salmonella*.

Besides chamber containing the study strains, a negative control with sterile buffer solutions was also considered in the assay.

Morphotype assays

Salmonella strains were grown in Luria-Bertani (LB) broth pH 7.0 – 7.2 (Tryptone 10 g, Difco; Yeast extract 5.0 g, Fluka; Sodium chloride 10.0 g, FagaLab, México, for 1 L) at 37°C overnight. Then, 10 µL of each culture were mixed with 10 µL sterile distillated water (SDW); aliquots of 3 µL were spot inoculated on LB agar supplemented with 40 µg mL^{-1} Congo red (Sigma-Aldrich, Germany) and 20 µg mL^{-1} Coomasie brilliant blue (Sigma-Aldrich, Germany) per duplicate and incubated at 28°C for 168 h. After incubation, bacterial growth was observed and measured in a stereoscopic microscope SAP08 Leica Microsystems (NC, USA) and classified within the morphotype RDAR, BDAR, PDAR and SAW (Anriany et al. 2001; Vestby et al. 2009) accordingly. *S.*Typhimurium catalogued as a RDAR morphotype was used as control strain and 3 µL of SDW as negative control. Morphotype growth (diameter) was measured every 24 h with a digital Vernier (Daigger Scientific, IL, USA).

Motility assays

Motility assay was conducted according to Rashid and Kornberg (2000) with some modifications. Briefly, plates of semi-solid culture medium prepared with LB $^{wo\ NaCl}$ and 0.3% agar were inoculated by deep puncture with a colony of *Salmonella* using a sterile straight nichrome handle (per duplicate) and incubated at 28°C for 8 h. As a negative control, a puncture in the agar with a straight sterile nichrome handle was used. Motility was evaluated by measuring the migration halo (mm) from the point of inoculation.

Biofilm formation

Biofilm formation was performed according to Stepanović et al. (2000) with slight modifications. Briefly, an overnight culture of LB of each *Salmonella* strain was diluted in Luria Bertani broth without salt (LB $^{wo\ NaCl}$) to an optical density of 600 nm (OD_{600}) = 0.2, then 30 µL of this suspension was transferred to 96-well polystyrene microtiter plates (Nunclon, Thermo Fisher Scientific, MA, USA), which contained 130 µL of LB $^{wo\ NaCl}$ and incubated statically for 96 h at 28°C. After incubation, the supernatant was discarded, and plates were washed three times with 200 µL of SDW

by gently rotary movements, moisture excess was removed inverting plates and tapping on them vigorously with an absorbent paper. Subsequently, adhered cells were fixed at 80°C for 15 min and stained with 130 µL of 1% crystal violet for 30 min. Then, microplate cells contained were solubilized with 130 µL of 33% (v/v) glacial acetic acid, incubated for 10 min at room temperature. The $OD_{595\ nm}$ of this solution was measured in a microplate reader Synergy HT spectrophotometer (Synergy HT, Bio-Tek Instruments, Inc., Winooski, VT, USA). Based on $OD_{595\ nm}$ results, strains were classified into four categories as no biofilm producers, weak, moderate or strong biofilm producers as previously described by Stepanović et al. (2000) and Díez-García et al. (2012).

Pellicle formation at liquid-air interface

Biofilm formation in microcosms was performed according to Constantin (2009) and Solano et al. (2002) with some modifications. One overnight culture (0.5 mL) was inoculated into 4.5 mL of LB $^{wo\ NaCl}$. The tubes were incubated statically at 28°C for 96 h. After incubation, tubes were subjected to visual observation; film growth surface coverage was categorized according to the following scale: 0 not visible, 1 appearance of a film on the surface of the medium, 2 very thin film on the surface of the medium, 3 film on the walls and on the surface of the medium, 4 rough film on the surface of the medium.

Pellicle resistance at liquid-air interface

Biofilm strength at the liquid–air interface was measured by means of maximum mass deformation (MMD). For this, glass beads (0.0338 g each) were carefully placed in the center of the film-formed on the microcosm top; the weight required for the biofilm to break or move to the bottom of the microcosm was recorded. Results were expressed in grams, multiplying the weight and number of glass beads that were required to break the pellicle (Spiers et al. 2006).

Cellulose detection by calcofluor method

Cellulose detection was analyzed with calcofluor white 200 mg mL^{-1} (Fluorescent Brightener 28, Sigma Aldrich, Germany) by two methods: 1) in liquid–air interface in LB broth and 2) in LB agar (Römling and Rohde 1999; Solano et al. 2002). For method 1, a tube with 4.5 mL of LB $^{wo\ NaCl}$ were inoculated with 0.5 mL overnight culture; for method 2, 3 µL of a dilution of 1:1 of overnight culture were spot inoculated in LB agar plate; the tubes and plates were statically incubated at 28°C for 96 h. The detection of fluorescence was observed at 365 nm with UVGL-55 handheld UV lamp (UVP, Cambridge, UK). All assays were performed by duplicate.

Neutral sugars and cellulose quantification

Overnight cultures were densely seeded in LB agar plates and incubated at 28°C for 96 h for biofilm formation. After incubation, biofilm was collected and placed in 1.5 mL Eppendorf tubes. The biofilm was dried with 0.5 mL of 80% ethanol. The mixture was vortexed for 2 min and sonicated 20 min to lyse cells, then agitated again and centrifuged at 3500 *g* for 5 min; the supernatant was removed, and tubes were placed in an oven at 44°C for 24 h. After biofilm was ground in a porcelain mortar and stored at room temperature for further analysis.

To obtain cells in the planktonic state (used as a control), a characteristic colony of *Salmonella* was selected and inoculated in 300 mL of Luria Bertani broth (LB), incubated at 37°C for 18 h, then three washes were carried out with phosphate buffer pH 7.2 ± 0.2 to recover the bacterial pellet and dried with ethanol, following the protocol indicated above.

Neutral sugars composition was determined by the method of alditol acetates (Albersheim et al. 1967) with slight modifications. Three milligrams of dry biofilm or bacteria in planktonic

state (per duplicate) were hydrolyzed for 1 h at 121°C with 500 µL of 2 N trifluoroacetic acid (TFA) containing 100 µg mL^{-1} of myo-inositol as internal standard. The hydrolyzed fraction was recovered for derivatization and non-hydrolyzed pellet was rinsed with methanol for monosaccharide removal and dried for cellulose assays. The derivatization consisted of a reduction reaction with methanol and $NaBH_4$ at 2% in 1 N NH_4OH (1 h at 25°C). After evaporation, an acetylation reaction with acetic anhydride in the presence of 1-methyl imidazole as a catalyst was done (Blakeney et al. 1983). After complete removal of methanol through solubilization-evaporation cycles, the derivatized alditol acetates were recovered in chloroform, then evaporated and recovered in high purity acetone for its quantification by gas chromatograph (Agilent 7890B, Agilent Technologies, Santa Clara, CA, USA) with FID detector (250°C), DB-23 capillary column (30 m x 0.25 mm J&W Scientific, Folsom, CA, USA) at 210°C. Helium at constant flow (3 mL min^{-1}) was used as carrier gas. The concentration of neutral sugars was calculated by integration of peaks and determination of the corresponding molar values using response factors established with standard monosaccharides from calibration curves of rhamnose, fucose, arabinose, xylose, mannose, galactose, glucose and ribose (Sigma-Aldrich, St. Louis, MO, USA). The results were expressed as a percentage of dry weight (% Dry Wt).

The non-hydrolyzed sediment in the procedure mentioned above, was further treated with 67% H_2SO_4 in an ice bath for 5 h (cellulose testing) to complete the hydrolyzations process, the glucose released was assayed using anthrone 0.2% in concentrated H_2SO_4 (Yemm and Willis 1954). The concentration was calculated from standards of glucose (0–100 µg) and read at 620 nm in a spectrophotometer (Cary 60 UV-Vis, Agilent Inc., Santa Clara, California).

Total protein quantification

Total proteins were determined by the Bradford method (Bradford 1976) with some modifications. In this assay, fresh and dry biofilm were used, as well as bacterium in planktonic state. Approximately 10–20 mg of sample was weighed in a 1.5 mL Eppendorf tube and 200 µL of distilled water was added, vigorously vortexed, incubated at 95°C for 10 min, and centrifuged at 10,000 *g* for 5 min. After this, 50 µL of supernatant were diluted with 50 µL of distilled water and placed into a tube, following 1 mL of the Bradford reagent (diluted 1:5) was added to each of the samples, vortexed to finally read the absorbance at 595 nm. A calibration curve was performed using the bovine serum albumin (Sigma Aldrich, Germany) as reference protein.

Curli fimbriae detection

This trial was carried out according to Zhou et al. (2012); Reichhardt et al. (2015); Nicastro et al. (2019) with some modifications. One mL of cell culture with an OD_{600} of 3.0 was centrifuged at 10,000 *g* for 5 min to recover the pellet, then 1.2 mL of TE (10 mM Tris, 1 mM EDTA, pH 7.5) with 2% SDS (sodium dodecyl sulfate) was added, followed by an incubation at 95°C for 45 min. After this, the sample was centrifuged and washed three times with SDW. To solubilize CsgA, samples were briefly treated with 250 µL of formic acid in ice for 5 min and then were frozen before lyophilization. After removing the formic acid (FA), the pellets enriched in CsgA were electrophoresed on 15% sodium dodecyl sulfate (SDS)-polyacrylamide gel and blotted onto polyvinylidene difluoride membrane (Bio-rad Laboratories, Inc. CA, USA) using standard techniques with a anti CsgA 1:10,000 (Abmart, Shanghai, China) as primary antibody and anti-mouse IgG (whole molecule) peroxidase antibody produced in rabbit 1:1000 (Sigma-Aldrich, St. Louis, MO, USA) as secondary antibody. ECL Western Blotting Substrate (Pierce™, Thermofisher Scientific, Waltham, MA, USA) was used to increase chemiluminescence detection according to the protocol of the manufacturer. Also, the visualization of CsgA was performed by Dot blots technique.

Statistical analysis

A completely randomized experimental design was applied to all experiments. The statistical package Minitab 18 (State College, PA, USA) was used to carry out the data analysis. All parameters were compared using analysis of variance (ANOVA) followed by Tukey test for comparisons among groups. All trials were performed per triplicate.

Results

Morphotype

Results showed a dependent morphotype/serotype relationship. In this regard, *S.* Infantis Env S-285 and *S.* Infantis Cli S-304 expressed the BDAR morphotype, showing a spider net type structure with tight channels (Figure 1A). *S.* Saintpaul Env S-70 presented RDAR morphotype, which is easily observed with naked eye as dark red/violet. RDAR colonies appear dry and rough with irregular edges with wide and high channels (through which nutrients, water and waste circulate), that start from the center to the edge of the colony (Figure 1A). The widely reported RDAR has shown to have the characteristics of greater resistance, and therefore greater probability of surviving in a non-host environment.

Interestingly, the environmental and clinical isolates of *S.* Oranienburg S-76 and S-347, presented a new morphotype. The softness, pink color and mucoid characteristics of the film showed in Congo red are unique and do not match with any of the previously reported morphotype in the literature; therefore, we propose the discovery of a new morphotype for *Salmonella* that according with its characteristics was named as SPAM (soft, pink and mucoid) (Figure 1). This nascent morphotype is composed of cellulose and curli fimbriae with an overexpression of polysaccharides.

Morphotype results showed some differences among serotypes and conditions they were exposed to. All strains when exposed to river water developed biofilms with an overlapping mucoid layer, starting from the center to the edge of the colony (Figure 1B).

At 96 h of growth, the well-defined morphotype was clearly observed (Figure 2A) and at 168 h morphotype growth of *Salmonella* strains (Figure 2B) had a significant difference with p values of 0.008 under exposure of *Salmonella* strains between scenarios, being larger when the strains were exposed to river water (Table 1). *Salmonella* Typhimurium ATCC 14028 was the strain with the

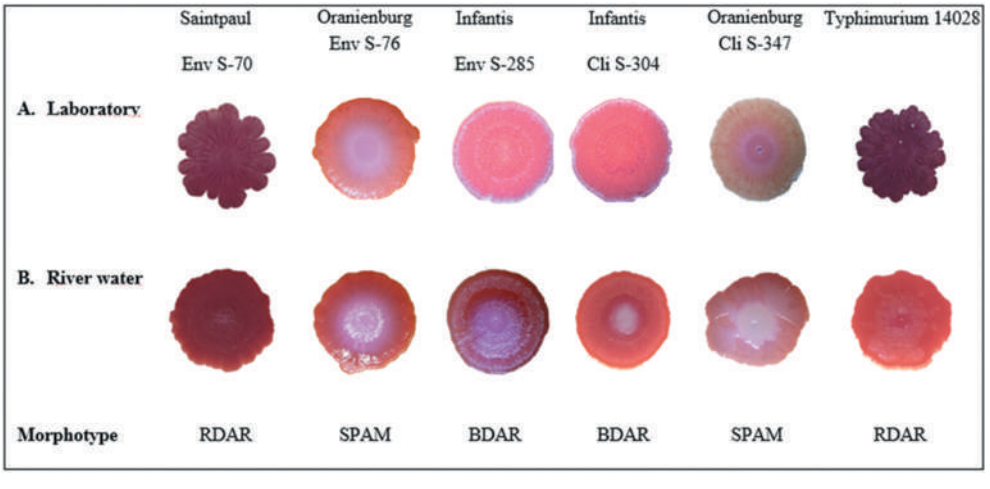

Figure 1. *Salmonella* morphotype. Differences under laboratory (A) and river water (B) exposition. Under river water exposition, the formation of a mucoid layer on the morphotype in all strains is observed. Morphotype classification (C). Cli=strains of clinical isolate, Env=strain of environmental isolate.

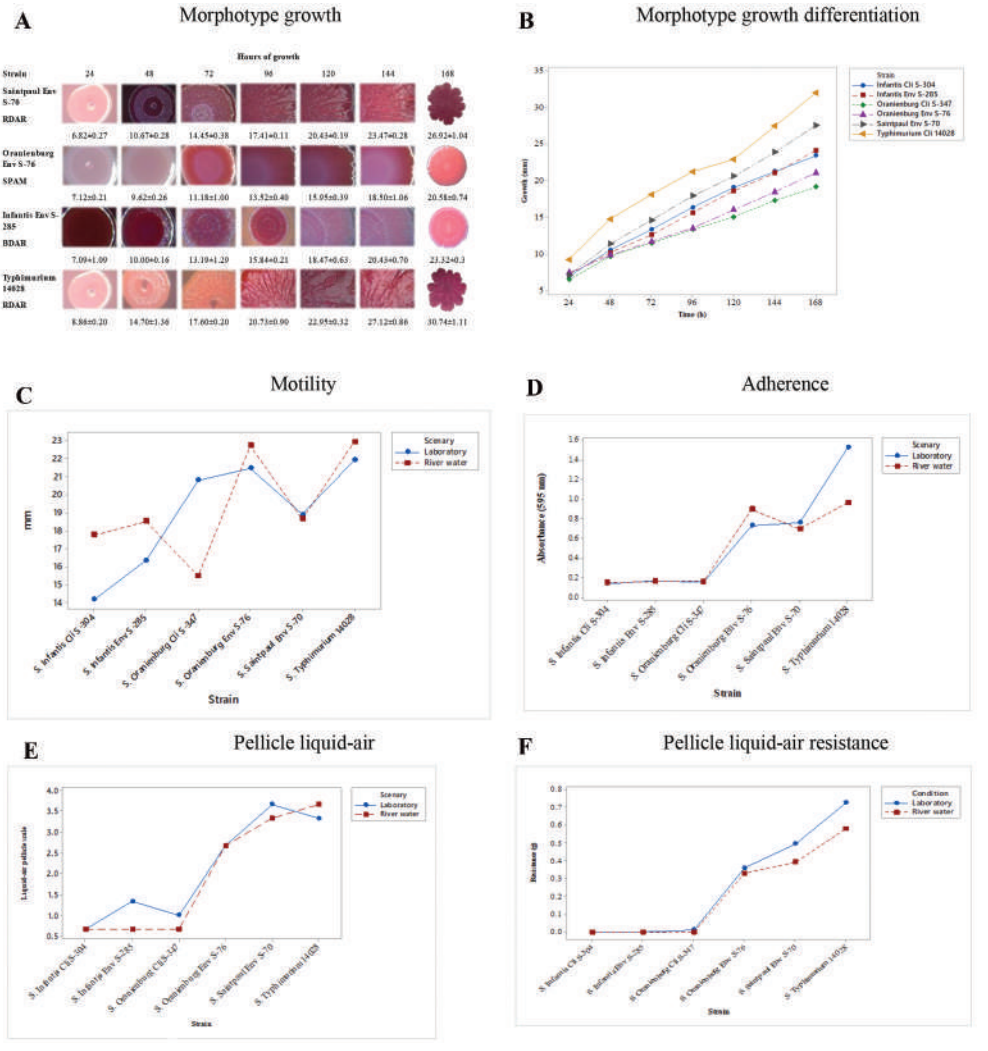

Figure 2. *Salmonella* strains behaviour under different scenarios (laboratory and river water exposition). (**A**) Morphotype growth at 168 h, (**B**) Morphotype growth differentiation at 24-168 h, (**C**) Strain motility in mm, (**D**) Adherence of *Salmonella* strains biofilm, (**E**) Pellicle liquid-air and (**F**) Pellicle liquid-air resistance. Cli= strains of clinical isolate, Env= strains of environmental isolate.

highest growth in both scenarios (30.74 ± 1.1 mm for laboratory and 33.31 ± 1.26 mm for river water), followed by environmental *S.* Saintpaul S-70 (26.92 ± 1.04 for laboratory and 28.25 ± 0.73 mm for river water). Concerning, *S.*Oranienburg strains of both origins showed the lowest growth (20.58 ± 0.74 mm and 18.35 ± 0.38 mm for laboratory and river water, respectively).

Morphotype differentiation, based on growth and measured every 24 h, was observed in the first hours of growth in the RDAR and BDAR morphotypes, not so for the SPAM morphotype, which presented a mucoid structure over time (Figure 2A). In this sense, growth in terms of morphotype, depending on the origin of isolation, did not cause any difference in morphotype among strains of the same serotype.

Table 1. Morphotype growth in diameter through time on *Salmonella* strains under different scenarios.

		Strains					
Scenary	**Hours**	**Saintpaul Env S-70** [b]	**Oranienburg Env S-76** [d]	**Infantis Env S-285** [c]	**Infantis Cli S-304** [c]	**Oranienburg Cli S-347** [e]	**Typhimurium Cli 14028** [a]
Laboratory [B]	24	6.82 ± 0.27	7.12 ± 0.21	7.09 ± 1.09	6.76 ± 0.54	6.14 ± 0.52	8.86 ± 0.20
	48	10.67 ± 0.28	9.62 ± 0.26	10.00 ± 0.16	10.05 ± 0.06	9.74 ± 0.43	14.70 ± 1.36
	72	14.45 ± 0.38	11.18 ± 1.00	13.19 ± 1.29	13.09 ± 0.78	11.21 ± 0.11	17.60 ± 0.20
	96	17.41 ± 0.11	13.52 ± 0.40	15.84 ± 0.21	15.82 ± .048	13.12 ± 0.86	20.73 ± 0.90
	120	20.43 ± 0.19	15.95 ± 0.39	18.47 ± 0.63	18.53 ± 0.40	14.65 ± 0.40	22.95 ± 0.32
	144	23.47 ± 0.28	18.50 ± 1.06	20.43 ± 0.70	20.96 ± 0.10	17.41 ± 1.56	27.12 ± 0.86
	168	26.92 ± 1.04	20.58 ± 0.74	23.32 ± 0.3	22.51 ± 1.50	18.35 ± 0.38	30.74 ± 1.11
River water [A]	24	7.46 ± 1.09	7.82 ± 0.78	7.40 ± 1.07	7.13 ± 1.36	6.85 ± 0.16	9.65 ± 0.31
	48	12.11 ± 1.01	10.11 ± 0.23	10.4*0 ± 0.43	11.08 ± 0.09	9.62 ± 0.73	14.88 ± 1.47
	72	14.78 ± 1.14	12.26 ± 0.97	12.03 ± 0.86	13.65 ± 0.28	11.82 ± 0.34	18.62 ± 0.59
	96	18.47 ± 1.55	13.57 ± 0.33	15.43 ± 1.23	16.90 ± 0.89	13.56 ± 1.03	21.79 ± 1.15
	120	20.83 ± 0.52	16.12 ± 0.80	18.83 ± 0.58	19.69 ± 0.58	15.51 ± 0.87	22.93 ± 0.88
	144	24.37 ± 1.12	18.46 ± 0.45	21.73 ± 0.39	21.63 ± 0.56	17.27 ± 1.14	27.82 ± 0.63
	168	28.25 ± 0.73	21.64 ± 0.56	25.05 ± 0.54	24.41 ± 1.27	19.98 ± 1.22	33.31 ± 1.26

Values mean in mm ± standard deviation ($n = 3$). Statistical differences between strains are indicated with lower case and differences between scenarios are in upper case ($p = 0.05$ by Tukey test). The differences are for columns. Cli = strains of clinical isolate, Env = strains of environmental isolate.

Motility

Motility results showed a similar behavior among strains regardless origin. Nevertheless, strains exposed to river water showed greater motility, except *S.* Oranienburg Cli S-347 (Figure 2C). *Salmonella* Saintpaul Env S-70 motility was from 18.91 ± 3.51/18.66 ± 0.90 mm, Oranienburg Env S-76 from 21.48 ± 3.36/22.73 ± 0.57 mm, Infantis Env S-285 from 16.36 ± 1.22/ 18.53 ± 2.40 mm, Infantis Cli S-304 from 14.19 ± 0.72/17.78 ± 3.24 mm, Oranienburg Cli S-347 from 20.80 ± 2.72/15.48 ± 2.79 mm and Typhimurium 14028 from 21.91 ± 1.70/22.93 ± 1.54 mm under laboratory and river water exposition, respectively. The most motile strains were Oranienburg Env S-76 and Typhimurium 14028; meanwhile the least motile strains were Infantis Env S-285 and Infantis Cli S-304. Based on results, our study suggests that, except Oranienburg Cli S-347, exposition to river water did not negatively affect the motility of *Salmonella* strains of either environment or clinical isolate, given that all strains maintained or increased their mobility when exposed to river water (Figure 2C).

Biofilm formation

Quantification of biofilm under different scenarios (laboratory and river water) is shown in (Figure 2D) measured as adherence. For *Salmonella* Saintpaul Env S-70 OD was from 0.624 ± 0.22/ 0.691 ± 0.11, Oranienburg Env S-76 OD from 0.651 ± 0.17/0.781 ± 0.20, Infantis Env S-285 from 0.165 ± 0.06/0.160 ± 0.02, Infantis Cli S-304 from 0.137 ± 0.01/0.145 ± 0.02, Oranienburg Env S-347 from 0.150 ± 0.03/0.155 ± 0.03 and Typhimurium 14028 from 1.525 ± 0.10/1.006 ± 0.13 under laboratory and river water exposition, respectively.

According to the classification of Stepanović et al. (2000), based on ODc (optical density of negative control) and OD (optical density of each strain), it was found that *Salmonella* Saintpaul Env S-70, *S.* Oranienburg Env S-76 and Typhimurium 14028 strains were strongly biofilm producers, contrarily to Infantis Env S-285, Infantis Cli S-304 and Oranienburg Cli S-347 strains, which were classified as weak biofilm producers; no matter the scenario they were exposed to.

Pellicle formation at liquid-air interface

Morphological characteristics, such as surface geometry, density of pellicle liquid-air after 96 hours of incubation at 28°C were scored from 0 to 4 as mentioned in materials and methods section. The total value of each strain corresponds to an average of three replicates performed in Minitab 18 statistical package. Results show that *Salmonella* Saintpaul Env S-70, Oranienburg Env S-76 and Typhimurium 14028 formed a pleated pellicle covering the entire surface of medium (3.667 ± 0.577, 2.667 ± 0.577 and 3.333 ± 0.577, respectively). Statistical analysis indicates no significant differences (p = 0.700) among the three strains when exposed to different scenarios (laboratory and river water). The weak biofilm producer strains (Infantis Env S-285, Infantis Cli S-304 and Oranienburg Cli S-347) received a score close to 1 meaning film appearance on the surface medium, with values 1.33 ± 0.577, 0.667 ± 0.577 and 1.000 ± 0.000, respectively, under laboratory exposition; meanwhile under river water exposition, these strains formed a very weak pellicle at the liquid–air interface corresponding to less than 1 with a score of 0.667 ± 0.577 for each one, which indicates the formation of a biofilm with weak interactions when exposed to non-host conditions (Figure 2E).

Pellicle resistance at liquid-air interface

Results for pellicle strength at the liquid–air interface showed significant differences by variance analysis with p value of 0.00 for factors strain and scenario. Under laboratory condition, the strain with major resistance was *Salmonella* Typhimurium 14028 with 0.726 ± 0.047 g, followed by Saintpaul Env S-70 and Oranienburg Env S-76 (0.494 ± 0.038 and 0.360 ± 0.016 g, respectively)

(Figure 2F). *Salmonella* Infantis Env S-285 showed null stress resistance (values of 0) and similar results for Infantis Cli S-304 and Oranienburg Cli S-347. The strains under river water exposure decreased slightly their film´s resistance showing 0.579 ± 0.024, 0.392 ± 0.079 g and 0.329 ± 0.061 g for Typhimurium 14028, Saintpaul Env S-70 and Oranienburg Env S-76, respectively.

Cellulose quantification

Biofilms formed under laboratory, river water exposition and bacteria in planktonic state were analyzed for cellulose content. Results show that cellulose was produced and detected in all biofilms formed when strains were exposed to the different scenarios (Figure 3A) with significant differences (p = 0.00) for the factors: strain, scenario and strain*scenario. *Salmonella* Typhimurium 14028 produces 2.718 ± 0.146 mg g^{-1}, 2.467 ± 0.185 mg g^{-1} and 0.951 ± 0.061 mg g^{-1} of cellulose; followed by Saintpaul Env S-70 with 1.655 ± 0.305 mg g^{-1}, 1.466 ± 0.233 mg g^{-1}, 0.780 ± 0.334 mg g^{-1} at laboratory, river water exposition and planktonic state. The lowest amount of cellulose produced was the strain Oranienburg Cli S-347 with 0.798 ± 0.140 mg g^{-1}, 0.823 ± 0.217 mg g^{-1}, 0.728 ± 0.144 mg g^{-1} of cellulose in each of the above mentioned scenarios, respectively.

Cellulose detection

All strains showed fluorescence with calcofluor white under UV light, indicating the presence of cellulose in pellicle liquid-air and Luria Bertani agar. The strains with a strong fluorescence were *Salmonella* Saintpaul Env S-70, Oranienburg Env S-76 and Typhmiurium 14028, while the strains with a weak fluorescence were Infantis Env S-285, Infantis Cli S-304 and Oranienburg Cli S-347 (Data not shown), coinciding with the weak biofilm-forming strains. These results are significant enough to differentiate exopolysaccharide production, such as cellulose in these forming biofilms strains.

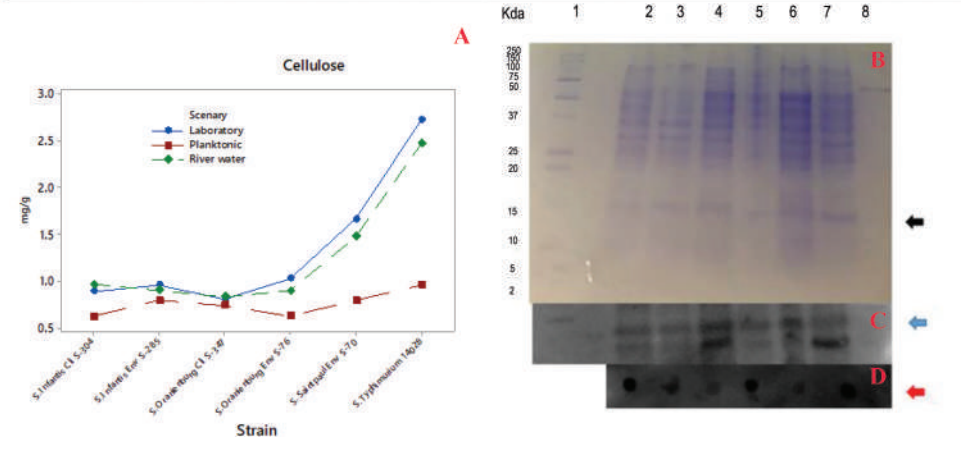

Figure 3. *Salmonella* biofilm composition. (**A**)Cellulose in *Salmonella* strains under different scenarios (laboratory, river water and planktonic state) at 28 ºC for 168 h. (**B**, **C** and **D**) Curli protein detection of *Salmonella* biofilms. (**B**) Polyacrylamide gel stained with Coomassie blue, lane 1: Molecular weight 2-250 Kda, lane 2-7 *Salmonella* strains: Saintpaul Env S-70, Oranienburg Env S-76, Infantis Env S-285, Infantis Cli S-304, Oranienburg Cli S-347 and Typhimurium 14028 (positive control), lane 8: Bovine Serum Albumin 7 µL at 400 $mgmL^{-1}$ as an internal control of protein. The bands are visualized at 17.5 Kda where the formic acid resistant CsgA fraction of curli is expected (black arrow). (**C**) Western blot and (**D**) Dot-blot with anti-CsgA antibodies showing CsgA protein expression (blue and red arrow), respectively. The tests were performed independently in triplicate.

Table 2. *Salmonella* total protein quantification by the Bradford method in fresh, dry biofilm and planktonic state..

Strain	Fresh biofilm [B]	Dry biofilm [A]	Dry planktonic state [B]
Saintpaul Env S-70	13.31 ± 0.49[a]	33.35 ± 5.33 [a]	19.53 ± 0.90 [a]
Oranienburg Env S-76	15.55 ± 1.46 [a]	41.75 ± 3.50 [a]	22.75 ± 1.71 [a]
Infantis Env S-285	5.61 ± 0.12[b]	14.73 ± 0.67 [b]	9.00 ± 0.34 [b]
Infantis Cli S-304	5.57 ± 0.51[b]	17.70 ± 0.89 [b]	9.26 ± 0.58 [b]
Oranienburg Cli S-347	15.42 ± 0.42 [a]	35.66 ± 3.20 [a]	15.67 ± 0.91 [a]
Typhimurium 14,028	11.04 ± 0.55 [a]	35.59 ± 2.29 [a]	17.71 ± 0.90 [a]

Values mean in μg g^{-1} of total protein ± SEM from replicates of three independent experiments. Statistical differences between strains are indicated with lower case and differences between scenarios are in upper case ($p < 0.05$ by Tukey test). The differences are for columns. Cli = strains of clinical isolate, Env = strains of environmental isolate.

Total protein quantification

The amount of total proteins present in each strain is co-related to curli protein, which is produced in biofilms. (Table 2) shows that fresh biofilm contained the lowest amount of total protein (30–40%), compared to dry biofilm in all strains. Dry biofilm contained 43–61% more protein than bacteria in dry planktonic state with statistical differences ($p< 0.05$). Under all scenarios, *Salmonella* Oranienburg contained the highest total protein while *Salmonella* Infantis had the lowest content compared to the other strains.

Curli fimbriae detection

Curli are extracellular protein fibers of the amyloid type being very stable and resistant to detergents, pH and Protease K, therefore formic acid (FA) or hexafluoro-2-propanol (HFIP) is needed to dissociate curli fimbriae (Steenackers et al. 2012; Reichhardt et al. 2015). CsgA is the resistant part and the major structural subunit of curli, which can be mobilized in a SDS-PAGE gel with an expected fraction close to 17.5 Kda and can be detected by Dot blot or Western blot using anti-CsgA antibodies (Zhou et al. 2012; Bordeau and Felden 2014; Evans and Chapman 2014; Nicastro et al. 2019). Figure 3 shows the CsgA fraction in SDS-PAGE (B), Western blot (C) and Dot-blot (D). All strains evaluated produce curli fimbriae as part of the biofilm matrix. The content of CsgA being greater in RDAR morphotype of *Salmonella* Saintpaul and Typhimurium. This component is present in the new SPAM morphotype (Figure 3B, C and D, lanes 3 and 6).

Neutral sugars quantification

Neutral sugar quantification was determined to biofilms formed under two scenarios (laboratory and river water exposition), but also compared to the observed in bacteria in planktonic state; this would allow to know the amount of neutral sugars based on differences by distinctive biofilm formation. Arabinose, fucose and xylose were present in a smaller amount with values near to 0.00–0.08% Dry wt in all strains and conditions. *Salmonella* Infantis Cli S-304 and Infantis Env S-285 showed the lowest amount of total neutral sugars (Figures 4A and 4B), followed by Oranienburg Cli S-347 and Oranienburg Env S-76 (Figures 4C and 4D). The strains with the highest amount of neutral sugars were Saintpaul Env S-70 and Typhimurium 14,028 (Figures 4E and 4F), where S. Saintpaul Env S-70 had rhamnose (1.46, 1.21, 0.88% Dry Wt), mannose (2.01, 1.67, 1.16% Dry Wt), galactose (2.21, 1.88, 1.64% Dry Wt) and glucose (1.84, 1.81, 1.09% Dry Wt) for scenarios: laboratory biofilm, river water biofilm and planktonis state, respectively. *S.* Typhimurium 14,028 had rhamnose (1.50, 1.0, 0.51% Dry Wt), mannose (1.91, 1.54, 0.92), galactose (1.98, 1.51, 0.69% Dry Wt) and glucose (2.02, 1.47, 0.93% Dry Wt) for the scenarios: laboratory biofilm, river water biofilm and planktonis state, respectively.

Results for neutral sugar suggest a morphotype serotype-dependent pattern; *Salmonella* Infantis (Env S-285/Cli S-304), *Salmonella* Oranienburg (Env S-76/Cli S-347) and *Salmonella* Saintpaul/Typhimurium (Env S-70/S-14028) (Figure 4G). Mannose was highly detected in Oranienburg strains in all scenarios

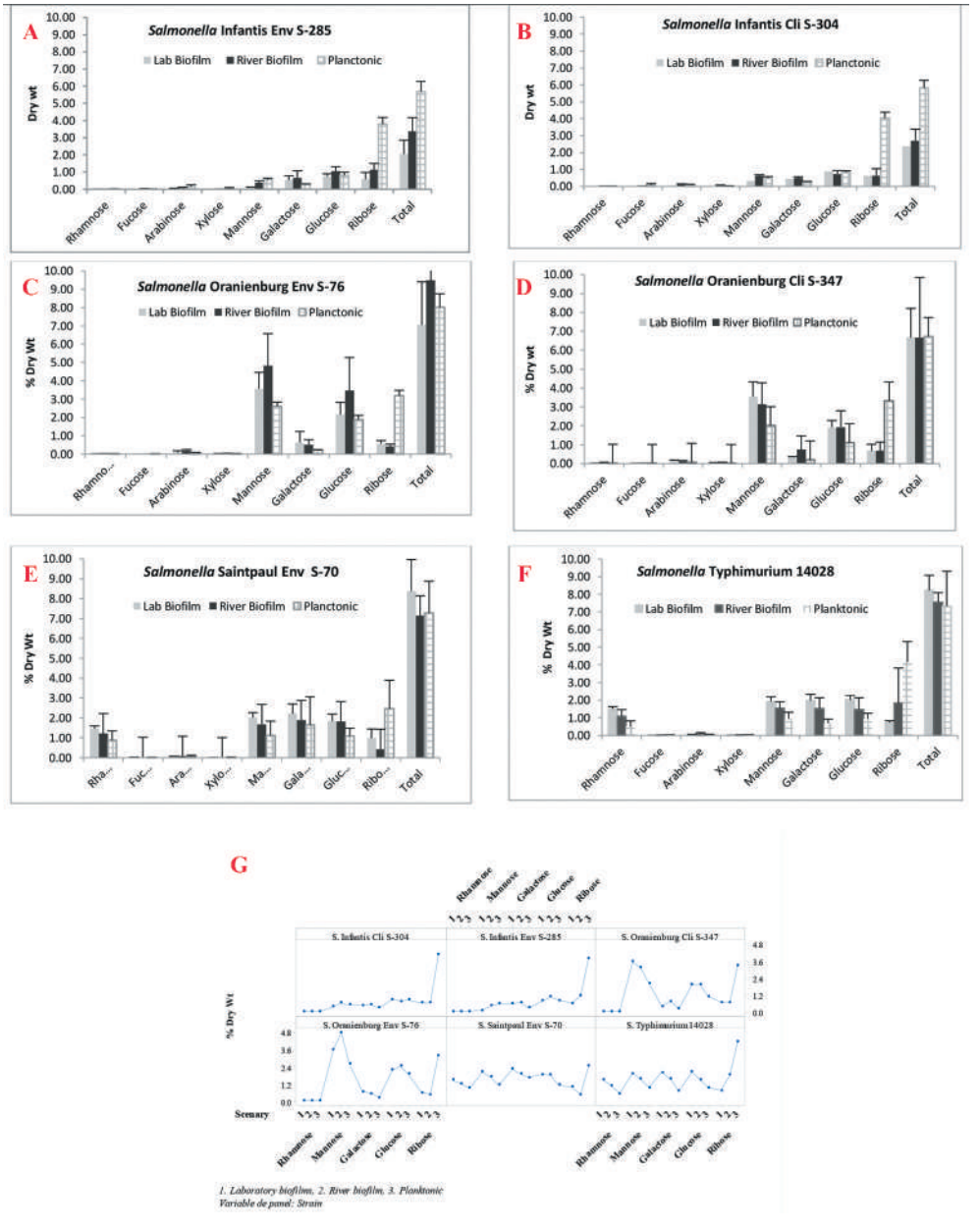

Figure 4. Neutral sugars composition of *Salmonella* strains under different scenarios (laboratory biofilm, river water biofilm and planktonic state). (**A**-**F**) *Salmonella* strains separately. (**G**) *Salmonella* strains together. The data represents mean of % Dry Weight of neutral sugars from replicates of three independent experiments.

studied with values for Oranienburg Cli S-347 (3.545, 3.130, 2.008% Dry Wt) and Oranienburg Env S-76 (3.57, 4.81, 2.61% Dry Wt) for laboratory biofilm, river water biofilm and planktonic state, respectively, being higher in Oranienburg Env S-76 when exposed to river water (4.81% Dry Wt).

The presence of ribose (pentose sugar) was detected in all strains and conditions, being higher in the planktonic bacteria, with values of 2.45 to 4.16% Dry Wt. Typhimurium 14028 and Infantis Cli S-304 strains had the highest amount of this sugar (4.16 and 4.04% Dry Wt) that raised values close to 0.5–1.82% Dry Wt in biofilms (Figure 4 A-G).

Discussion

In surface water, everything that lives in it depends upon its own survival strategies. Biofilms are the vital protective measure of vegetative bacteria. *Salmonella* in planktonic state is highly affected by the characteristics of the binding surface and the physicochemical conditions that surround it. There is a vast scientific works detailing the resistance mechanisms deployed by *Salmonella* to counteract adverse environmental conditions (Solano et al. 2002; White and Surette 2006; Steenackers et al. 2012); surprisingly, biofilms structure and composition are not quite well described yet specially when the bacteria are exposed to such type of environments (Gaertner et al. 2011; Sha et al. 2011). Therefore, the present study not only provides evidence of *Salmonella*´s behavior in a non-host environment such as river water, but also provides knowledge about composition and structure of biofilm forming process under this scenario.

In our study, environmental and clinical strains showed morphological and compositional biofilm differences when faced with both laboratory and river water conditions. To the best of our knowledge, this is the first report of the expression of extracellular matrix components from environmental and clinic *Salmonella* isolates exposed to river water.

At the first phase of this investigation the RDAR morphotype was detected in the environmental strain *S.* Saintpaul S-70, which according to literature this morphotype is the most resistant of all morphotypes to date described, *S.* Saintpaul S-70 behavior was similar in all tests carried out to *S.* Typhimurium 14028 strain. The composition of the widely described RDAR morphotype was cellulose, curli and a high total protein content and neutral sugars. These biofilms components give the strain greater resistance, which allows the bacterium to remain viable for prolonged periods of time in the environment (Steenackers et al. 2012).

Moreover, both environmental and clinical Infantis strains with BDAR morphotype generated weaker adhesion capacity and liquid–air pellicle than other well-defined morphotypes. It should be noted that what has been reported so far about *Salmonella* morphotypes composition, describe that the BDAR morphotype is only composed of curli fimbriae with no cellulose expression, but in this research, we detected small amounts of cellulose production in BDAR morphotype. Thus, *Salmonella* Infantis produces weak biofilms that relate to the low level of the structural components (Steenackers et al. 2012). Even so, some *Salmonella* serotypes, with BDAR morphotype, can form a fragile matrix of exopolysaccharides, but unable to form a mature biofilm (Malcova et al. 2008; Medrano-Félix et al. 2018), coinciding with our results; even though some others have reported that BDAR morphotype in *Salmonella* Infantis or Agona are a good biofilm producers (Vestby et al. 2009; Aksoy 2019). Also, the results of the present study state that differentiating the BDAR from the RDAR morphotype based only on the characteristics of the colony might not be accurate. BDAR morphotype formed by Infantis strains produced curli and traces of cellulose; these two substances interact with the Congo red dye, so the BDAR morphotype appears reddish brown, not agreeing with the brown color of BDAR morphotype. This indicates that more studies are needed to compare morphotype characteristics according to the environment, to elucidate behavior patterns in relation to biofilm composition.

Results from the environmental and clinical Oranienburg strains (S-76 and S-347) were remarkable. Both isolates presented a non-previously reported morphotype; and according to its visible pellicle characteristics it was named as SPAM. Most of the reports about bacterial morphotypes describe RDAR, PDAR, BDAR and SAW (Solano et al. 2002; White and Surette 2006; Steenackers et al. 2012) as the common morphotype in *Salmonella* strains, while other morphotypes such as SBAM (soft, brown and mucoid) and BAS (brown and smooth) are less common (Malcova et al. 2008; Ramachandran et al. 2016). Bokranz et al. (2005) detected diverse morphotypes in *Escherichia coli* based on the expression of phenotype on Congo red agar composed by curli only, such as RAS (red and smooth) and BAS (brown and smooth) or cellulose only, PAS (pink and smooth) (Bokranz et al. 2005) and a mucoid phenotype was also reported in *E. coli* (Milanov et al. 2015). Contrarily, other studies in *Salmonella* have suggested to report as 'unidentified morphotype' in *Salmonella*

Agona and Infantis (Karaca et al. 2013; Aksoy 2019). SPAM morphotype reported here is composed of cellulose and curli fimbriae, with high content of mannose, followed by glucose and galactose. In this study, *Salmonella* Oranienburg from environmental origin (Env S-76) produces more resistant biofilms than its clinical counterpart (CliS-347), highlighting that these characteristics may have been acquired through generations exposed to non-favorable environmental conditions, which in turn has converted in a trait related and conserved for *Salmonella* and explains the natural resilience that this bacterium deploys as a survival strategy in natural settings. Additionally, we analyzed morphotype in ten additional strains of *Salmonella* Oranienburg isolated from river and canal water of which all presented the new morphotype SPAM (data not shown).

It was observed that strains exposed to river water expressed an overlapping mucoid layer, suggesting a diverse ability of *Salmonella* to form biofilms in the response to surrounding conditions in aquatic environment.

Results for motility showed that strains with the greatest movement capacity were Typhimurium (control strain), Oranienburg, Saintpaul and the least were the Infantis strains; however, when exposed to river water increased their motility, except for Oranienburg S-347; this indicates that clinical isolates are not able for a rapid response to adapt to non-host conditions. Some research indicates that adhesion factors, biofilm composition and cell motility are affected by environmental signals in different bacteria (Crawford et al. 2010; Chelvam et al. 2014; Rossi et al. 2018); although motile and non-motile species form biofilms, in motile species the ability to move using flagella or pili is generally an advantage for efficient adhesion to the cell surface (Rossi et al. 2018).

Adherence is another important factor to promote the ability of biofilm formation and its resistance. Pellicle liquid-air and its resistance expressed as maximum deformation of mass results showed that all strains under study had similar values in these trials and there were no significant differences to exposure to river water. Nonetheless, it was possible to observe that *Salmonella* Saintpaul Env S-70 and *S*. Oranienburg Env S-76 strains were strongly biofilm producers compared to Typhimurium 14028 strain widely reported as a strong biofilm former; contrarily to Infantis Env S-285, Infantis Cli S-304 and Oranienburg Cli S-347 strains, which were classified as weak biofilm producers no matter the scenario they were exposed to. This concur with several studies which suggest that bacterial isolates highly producers of curli and cellulose are more able to adhere and to survive on living or inert surfaces (Jonas et al. 2007; Vestby et al. 2009; Steenackers et al. 2012). It is interesting to highlight the behavior of both Oranienburg strains (S-76 and S-347), where S-76 is an environmental isolate strain (river water) and according to Medrano-Félix et al. (2018) suggest that *Salmonella* strains of environmental origin show better survival and ability to adapt to non-host conditions such as river water (Medrano-Félix et al. 2018).

Gram-negative bacteria contain thousands of proteins that are transported to the cell surface where they perform their specific functions (Paulsen et al. 1997), such as bacterial pathogenicity and biofilm formation, host recognition or nutrient acquisition (Dalbey and Kuhn 2012); the stable connection between bacteria and substrate surface is maintained by specific cell membrane proteins, as cell appendages such as flagella, fimbria, pili and secretion proteins called adhesins (Steenackers et al. 2012; Yaron and Römling 2014; Tilahun et al. 2016). González-Machado et al. (2018) detected proteins and β-polysaccharides as predominant components inside the EPS matrix of *S*. Agona biofilms, being the proteins the greatest biovolume among the extracellular components within biofilms which contribute to the biofilm architecture and may be used to identify biofilm formation (Gonzalez-Machado et al. 2018). In our study *Salmonella* Oranienburg had the highest total protein content and *Salmonella* Infantis the lowest compared to the other strains, but all strains evaluated produced curli fimbriae as part of the biofilm matrix with the presence of CsgA.

On the other hand, results for neutral sugar content showed that *Salmonella* Infantis Cli S-304 and Env S-285 had the lowest amount, followed by Oranienburg Cli S-347 and Oranienburg Env S-76. The strains that had the highest amount of neutral sugars were Saintpaul Env S-70 and Typhimurium 14028 with rhamnose, mannose, galactose, glucose in similar quantities. Few studies have shown the presence of polysaccharides in *Salmonella* biofilms, for instance, it was shown that

the main components of capsular polysaccharides in *S.* Typhimurium DT104 in biofilms were glucose, mannose and galactose (De Rezende et al. 2005) and also were detected these sugars at high levels as final products of gluconeogenesis, due to the high demand for exopolysaccharides production in biofilm formation (White et al. 2010). Others reported SBAM morphotype, whose composition depends solely on an overproduction of capsular polysaccharide because they produced neither cellulose nor curli fimbriae (Malcova et al. 2008). These capsular polysaccharides are important in adhesion elements for strains that do not express curli or cellulose and are capable of forming biofilms; even though others highlighted that cellulose does not appear to be a major component in *Salmonella* biofilms (Vestby et al. 2009).

A morphotype dependent behavior was observed in the type and amount of neutral sugars present for each strain. Mannose was highly detected in Oranienburg strains in all scenarios studied being higher in Oranienburg Env S-76 when exposed to river water.

The presence of ribose, was detected in all strains and in all conditions, being higher in planktonic bacteria, where Typhimurium 14028 and Infantis Cli S-304 were the strains that had the greatest amount of this compound. The 5-carbon sugar ribose is an important component of nucleotides and is found in RNA; it is known that within the biofilm formation there are cells in the planktonic state and cells segmented to the exopolysaccharide matrix; this might explain the detection of ribose as a component of biofilms in this study. Interestingly, diverse studies demonstrated that D-ribose inhibited AI-2 (autoinducer 2) that induced biofilm growth and co-aggregation in Gram negative and Gram positive bacteria (Jang et al. 2013; Lee et al. 2015; Cho et al. 2016; Sintim and Gürsoy 2016; Liu L et al. 2017). There is no information about the role of ribose in *Salmonella* biofilms; however, the literature shows ribose as a biofilm inhibiting agent; therefore, these findings open an important source of opportunities to expand scientific search for knowledge about biofilm formation and composition in *Salmonella* and other enterobacterias.

Conclusions

The present work appears to be the first to present a comparison biofilm-forming abilities and composition of both environmental and clinical *Salmonella* isolates and the effect of the environment on the ability to form biofilms during their exposure to river water as a survival strategy.

A new morphotype (SPAM) not previously reported was observed in *Salmonella* Oranienburg, where environmental and clinical isolates had differences in morphotype composition and resistance.

The results of this study contribute to determine the effect of non-host environment exposure on the composition of biofilms having a broader approach to the biofilm formation characteristics of *Salmonella*, and consequently to establish strategies that contribute to the prevention of these bacterial communities in the water and therefore in the food industry. This research shows relevant information that includes the ability of *Salmonella* to deploy one of the most powerful survival strategies, biofilm formation, as means for including itself as a component of the ecosystem and not only as a transitory contaminant.

It is recommended to extend the exposure time into river water and to conduct genomic studies in these strains to widen the knowledge and have a better and detailed understanding about the characteristics that make morphotype different among serotypes.

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Conflicts of Interest

'The authors report no conflict of interest.'

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