Biofilm Formation by Multidrug-Resistant Serotypes of *Salmonella* Isolated from Fresh Products: Effects of Nutritional and Environmental Conditions

María-Guadalupe Avila-Novoa, Pedro-Javier Guerrero-Medina, Velia Navarrete-Sahagún, Itzel Gómez-Olmos, Noemí-Yolanda Velázquez-Suárez, Lucía De la Cruz-Color, and Melesio Gutiérrez-Lomelí

Abstract: *Salmonella* serotypes can develop biofilms in fresh food products. This study focused on determining the antimicrobial resistance profile and the effects of different growth media and environmental conditions on biofilm formation by multidrug-resistant serotypes of *Salmonella*. All 49.4% of the *Salmonella* strains (five serotypes) were multidrug resistant. Assessment of the ability to form biofilms using the crystal violet staining method revealed that 95.6% of the strains of *Salmonella* were strong biofilm producers in 96-well polystyrene microtiter plates. Overall, 59.3% of the strains showed the rdar (red dry and rough colony) morphotype, 2.1% pdar (pink dry and rough colony), 27.4% bdar (brown dry and rough colony) and 10.9% saw (smooth and white colony), at two temperatures (22 and 35 °C). Mono-species biofilms of *Salmonella* serotypes showed a mean cell density of 8.78 log_{10} CFU/cm^2 ± 0.053 in TSBS (1/20 diluted TSB (tryptic soy broth) + 1% strawberry residues) and 8.43 log_{10} CFU/cm^2 ± 0.050 in TSBA (1/20 diluted TSB + 1% avocado residues) on polypropylene type B (PP) (p < 0.05). In addition, epifluorescence microscopy and scanning electron microscopy (SEM) enabled visualizing the bacteria and extracellular polymeric substances of biofilms on PP.

Keywords: *Salmonella;* serotypes; biofilm; organic matter; food surface contact

1. Introduction

*Salmonella* spp. cause about 1.35 million infections, 26,500 hospitalizations, and 420 deaths annually in the United States; Enteritidis, Newport, Typhimurium, Javiana, 4,5,12:i and Infantis were the top six *Salmonella* serotypes that caused foodborne infections in the United States in 2019 [1]. According to the Centers for Disease Control and Prevention, from 2006 to 2019, foodborne outbreaks of the *Salmonella* serotypes Newport, Infantis, and Typhimurium were linked to the consumption of fresh products with maradoll papaya, cucumber, cantaloupe, avocado, tomato, frozen raw tuna, and other products in the United States [2]. The ability of *Salmonella* serotypes to form biofilms has been demonstrated on stainless steel and polyethylene food contact surfaces or on polystyrene, glass, plastic, cement, and rubber [3,4]. In fact, biofilms formed in food processing environments are a constant source of microbial contamination that may lead to food spoilage or contamination.
foodborne diseases [5]. Biofilm cells promote biocorrosion of equipment, which promotes cross-contamination and reduced susceptibility to disinfectants [6].

Resistant Salmonella serotypes are considered a global public health problem that affect the food chain. Each year in the United States, there are an estimated USD 400 million of direct medical costs connected to the serotypes Salmonella typhi and Salmonella non-typhoidal [7].

However, Salmonella genomic island 1 (SGI1) is associated with multiple drug resistance (MDR); it has been demonstrated that SGI1 has an additional positive effect on biofilm formation [8,9]. Briefly, curli fimbriae and cellulose are components of the extracellular polymeric matrix produced by Salmonella, making them tolerant to disinfection processes and resistant to antimicrobial treatments or desiccation [10–12]. When cellulose synthesis is associated with the presence of curli fimbriae, Salmonella produce the red, dry, and rough (rdar) morphotype on Luria–Bertani agar supplemented with Congo red [3,13,14].

Additionally, the adhesion and biofilm-forming ability of Salmonella are determined by the physiochemical properties of cells, bacterial structures (pili, curli, fimbriae, flagella, and surface lipopolysaccharides), growth medium, type, surface characteristics, the presence of organic matter, and environmental factors [15,16]. Hence, the objectives of this research were (i) to determine the antimicrobial resistance profile and morphotype of the Salmonella serotypes isolated from fresh fruits and (ii) to evaluate the effects of different growth media and environmental conditions on biofilm formation by multidrug-resistant serotypes of Salmonella on polypropylene food contact surfaces.

2. Materials and Methods

2.1. Bacterial Strains

A total of 91 strains of Salmonella (five serotypes: Salmonella Rubislaw (n = 17), Salmonella Newport (n = 17), Salmonella Oranienburg (n = 3), Salmonella Infantis (n = 40), and monophasic Salmonella Typhimurium (n = 14)) were isolated from fresh fruits and characterized by Gomez-Olmos [17]. The isolation of Salmonella was carried out on xylose lysine desoxycholate (XLD) agar, Hektoen enteric (HE) agar, and bismuth sulfite (BS) agar (Becton Dickinson Bioxon, Le Pont de Claix, France); after incubation for 24–48 h at 35 °C, the isolates were further identified by conventional biochemical testing and serologic typing [18]. Stocks were stored in tryptic soy broth (TSB; Becton Dickinson Bioxon, Le Pont de Claix, France) containing 30% glycerol at −80 °C. Working cultures were maintained in TSB for 24 h at 37 °C to obtain a final concentration of 10^8 CFU/mL.

2.2. Antimicrobial Susceptibility Testing

Their patterns of resistance and/or susceptibility were determined using the agar diffusion method according to the American Clinical Laboratory Standardization Committee (CLSI) [19]. The antibiotics used were amikacin (AMK: 30 µg), ampicillin (AM: 10 µg), carbenicillin (CB: 100 µg), cephalothin (CF: 30 µg), cefotaxime (CFX: 30 µg), ciprofloxacin (CPF: 5 µg), chloramphenicol (CL: 30 µg), gentamicin (GE: 10 µg), netilmicin (NET: 30 µg), nitrofurantoin (NF: 300 µg), norfloxacin (NOF: 10 µg), and trimethoprim-sulfamethoxazole (SXT: 2.5/23.75 µg) (BBL™ Sensi-Disc™). The isolates were cultured on Mueller Hinton agar plates inoculated with a bacterial suspension equal to 0.5 McFarland and incubated at 37 °C for 24 h. The inhibition zone was measured after 24 h, and isolates were interpreted as susceptible (S), intermediate (I), or resistant (R) with reference to the standards set by the CLSI [19]. Salmonella Typhimurium ATCC 14028 was used as the positive control.

2.3. Determination of Morphotype

The morphotype of the Salmonella strains was determined according to the protocol described by Zogaj et al. [20] and Paz-Méndez et al. [21]. Working cultures were maintained in TSB for 24 h at 37 °C. Subsequently, a sub-culture of each Salmonella strain was carried out in Congo red agar (CRA) and incubated at 22 and 35 °C for 96 h. Briefly, CRA was prepared with Luria–Bertani (LB) broth without salt (Becton Dickinson Bioxon, Le Pont
de Claire, France), 15 g/L of bacteriological agar (Becton Dickinson Bioxon, Le Pont de Claire, France), adding 40 mg/L of Congo red (Sigma-Aldrich, Steinheim, Germany) and 20 mg/L of Coomassie brilliant blue (Sigma-Aldrich, Steinheim, Germany). The Salmonella morphotypes were interpreted into the following categories according to the macroscopic characteristics that developed in the CRA: (a) red dry and rough colony (rdr; express curli fimbriae and cellulose), (b) pink dry and rough colony (pdr; express cellulose), (c) brown dry and rough colony (bdr; express curli fimbriae), (d) smooth and white colony (saw) [20–22].

2.4. Development of Mono-Species Biofilms by Salmonella Serotypes

2.4.1. Polystyrene Biofilm Formation Assays

The ability of all Salmonella strains to form biofilms was tested in 96-well flat-bottomed polystyrene microtiter plates (Corning® 96-Well Assay Microplate, Lowell, MA, USA) using the crystal violet (CV) assay method [5]. For each strain, three wells of the microtiter plate were filled with 230 µL of 1/20 diluted TSB and 20 µL of bacterial suspension (~10⁸ CFU/mL). Then, the plates were incubated at 35 °C for 24 h. Wells filled with broth medium (1/20 diluted TSB) were used as negative controls, and Salmonella Typhimurium ATCC 14028 was used as the positive control. Next, the content of each well was aspirated and washed three times with phosphate-buffered saline (PBS; 7 mM Na₂HPO₄, 3 mM NaH₂PO₄, and 130 mM NaCl, pH 7.4; Sigma-Aldrich, Dorset, England) to remove planktonic bacteria. The attached bacteria were fixed with 250 µL of methanol (Sigma-Aldrich, St. Louis, MO, USA) for 15 min; then, the plates were emptied and left to dry. The plates were stained with 250 µL of 1% (w/v) crystal violet (CV; Hycel, Zapopan, Mexico) solution per well for 5 min. Excess stain was rinsed off with sterile distilled water, and the microtiter plates were air-dried. After that, adherent cells were resolubilized with 250 µL of 33% (v/v) glacial acetic acid (Sigma-Aldrich, St. Louis, MO, USA) per well. The optical density of each well was measured at 570 nm (OD₅₇₀) using a Multiskan FC (Thermo Fisher Scientific Inc., Madison, WI, USA). The cut-off OD (ODc) was defined as three standard deviations above the mean OD of the negative control. Based on the OD of bacterial films, strains were classified into the following categories: non-biofilm producers (OD ≤ ODc), weak biofilm producer (ODc < ODc ≤ (2 × ODc)), moderate biofilm producer ((2 × ODc) < OD ≤ (4 × ODc)), and strong biofilm producers ((4 × ODc) < OD), as previously described [5].

2.4.2. Conditions and Quantification of Biofilm Formation on Polypropylene Type B Surfaces

The mono-species biofilms were developed in vitro in two culture media: TSBS (1/20 diluted TSB + 1% strawberry residues) and TSBA (1/20 diluted TSB + 1% avocado residues) on PP coupons (polypropylene type B (2 × 0.7 × 0.2 cm; Plásticas Tarkus, Jalisco, Mexico)) at 35 °C for 240 h, with the replacement of the culture medium (TSBS or TSBA) at 120 h using the protocol described by Solis-Velazquez et al. [23]. After the incubation period, coupons were removed from the tubes under sterile conditions, and unbound cells were removed by vortexing (150 rpm for 10 s) with PBS. Each coupon was introduced individually into 5 mL of casein peptone (1 g/L; Becton Dickinson Bioxon, Le Pont de Claire, France), and the biofilms were removed by sonication (50–60 Hz for 1 min; Sonicor Model SC-100TH, West Babylon, NY, USA). Serial dilutions were performed and conventional plate counting was performed on tryptic soy agar (TSA; Becton Dickinson Bioxon, Le Pont de Claire, France) with incubation at 37 °C for 24 h. Three replicates were performed for each strain, and Salmonella Typhimurium ATCC 14028 was used as the positive control.

2.4.3. Epifluorescence Microscopy

After the incubation period of 240 h at 35 °C with the replacement of the culture medium (TSBS or TSBA) at 120 h, the PP coupons were removed and processed using the protocol described by Solis-Velazquez et al. [23]. The PP coupons were removed
and transferred to a tube containing 5 mL of PBS and vortexed for 10 s at 150 rpm to eliminate non-adhered cells. The coupons were stained with 0.025% acridine orange (AO; Sigma-Aldrich, St. Louis, MO, USA), rinsed with sterile distilled water, and dried in a level II cabinet (LABCONCO® Purifier® Biological Safety Cabinet, Kansas City, MO, USA). Biofilms were observed under a Nikon Eclipse E400 epifluorescence microscope using a 100× oil immersion lens and a blue BA 515 B-2A excitation filter at a 450–490 nm emission wavelength. The interpretation of the microscopic observations was based on the cells stained with acridine orange [24,25]. *Salmonella* Typhimurium ATCC 14028 was used as the positive control. As a negative control, a PP coupon without inoculum was included in all the assays.

2.4.4. Scanning Electron Microscopy (SEM)

After incubation at 35 °C for 240 h with the replacement of the culture medium (TSBS or TSBA) at 120 h, the PP coupons were treated as indicated in Section 2.4.3. They were further dried and transferred to 2% glutaraldehyde (DermoDex, Tlalpan, CDMX, Mexico) at 4 °C for 2 h to fix the sample. After serial dehydration in ethanol (30%, 50%, 60%, 70%, 90%, and 95%; Sigma-Aldrich, St. Louis, MO, USA) for 10 min each at 4 °C, every coupon was rinsed (three 10 min rinses) in 100% ethanol [26]. Samples were critical-point-dried and coated with gold for 30 s [27]. Biofilms were observed using a TESCAN Mira3 LMU scanning electron microscope (Brno—Kohoutovice, Czech Republic).

2.5. Statistical Analysis

All the experiments were evaluated using analysis of variance (ANOVA), followed by a least significant difference (LDS) test, in the Statgraphics Centurion XVI software program (StatPoint Technologies, Inc., Warrenton, VA, USA). Antibiotic resistances for each antibiotic agent were compared using a chi-square test or Fisher’s exact test when the expected value in any cell of the contingency table was less than 5, using IBM SPSS 21 software for Windows (IBM SPSS Corp., Armonk, NY, USA). Student’s t-test with SPSS 21 was used to analyze the data obtained by quantitative biofilm production assay. The results were expressed as means ± standard deviations (SDs) of three independent experiments. The confidence level was 95%, and the differences were considered statistically significant when *p* < 0.05. The Pearson correlation coefficient (PCC) was used for association between variables with SPSS 21.

3. Results

3.1. Antimicrobial Susceptibility Testing

The antimicrobial susceptibility testing results of the *Salmonella* strains (five serotypes) are shown in Figure 1. According to the results reported in Figure 1, 50.5% were resistant to ampicillin, 36.2% to carbenicillin, 31.8% to amikacin, and 19.7% to ciprofloxacin. Furthermore, there was a statistically significant difference in resistance between ampicillin and amikacin, and carbenicillin and ciprofloxacin (*p* < 0.05). On the basis of the obtained antibiotic resistance profiles, 49.4% of the *Salmonella* serotypes were multidrug resistant (Table 1).
Figure 1. Antimicrobial resistance pattern of *Salmonella* strains (five serotypes) to different antibiotics. AMK, amikacin; AM, ampicillin; CB, carbenicillin; CF, cephalothin; CFX, cefotaxime; CPF, ciprofloxacin; CL, chloramphenicol; GE, gentamicin; NET, netilmicin; NF, nitrofurantoin; NOF, norfloxacin; STX, trimethoprim-sulfamethoxazole. * Statistical significance at the $p < 0.05$ level.

Table 1. Characteristics of multidrug resistance of serotypes of *Salmonella* from fresh fruits.

| Strains                     | No. (%) of Isolates with Resistance | Resistance Profile          |
|-----------------------------|-------------------------------------|----------------------------|
| *Salmonella Rubislaw* (n = 17) | 4 (4.3)                             | AM-CB                      |
|                             | 1 (1)                               | AM-CB-GF-NF                |
|                             | 1 (1)                               | AK-CF-CL-NF                |
| *Salmonella Newport* (n = 17) | 3 (3.2)                             | AM-CB                      |
|                             | 1 (1)                               | AK-AM                      |
|                             | 1 (1)                               | AK-AM-CB                   |
|                             | 2 (2)                               | AK-CF-CPF-CL               |
|                             | 1 (1)                               | AK-CF-CPF-CPF-CL           |
| *Salmonella Oranienburg* (n = 3) | 1 (1)                             | AM-CB                      |
|                             | 1 (1)                               | AK-AM-CB                   |
| *Salmonella Infantis* (n = 40) | 7 (7)                              | AM-CB                      |
|                             | 2 (2)                               | AK-CPF                     |
|                             | 1 (1)                               | AK-CFX                     |
|                             | 2 (2)                               | AK-AM-CPF                  |
|                             | 1 (1)                               | AK-CPF-CL                  |
|                             | 1 (1)                               | AK-CFX-CPF                 |
|                             | 1 (1)                               | AK-AM-CB                   |
|                             | 1 (1)                               | AM-CB-CPF-CPF              |
|                             | 1 (1)                               | AK-CB-CPF-CPF              |
|                             | 1 (1)                               | AK-CF-CPF-CPF-CL           |
|                             | 1 (1)                               | AK-AM-CPF-CPF-CL           |
| *Salmonella Thphimurium* (n = 14) | 4 (4.3)                          | AM-CB                      |
|                             | 3 (3.2)                             | AK-AM-CPF                  |
|                             | 1 (1)                               | AK-CF-CPF                  |
|                             | 1 (1)                               | AK-AM-CB                   |

AMK, amikacin; AM, ampicillin; CB, carbenicillin; CF, cephalothin; CFX, cefotaxime; CPF, ciprofloxacin; CL, chloramphenicol; GE, gentamicin; NET, netilmicin; NF, nitrofurantoin; NOF, norfloxacin; STX, trimethoprim-sulfamethoxazole.
3.2. Ability to Form Mono-Species Biofilms

The mean OD values obtained by the quantitative biofilm production and morphotype of *Salmonella* strains (five serotypes) are shown Table 2. Overall, 95.6% of the *Salmonella* strains (five serotypes) were strong biofilm producers (mean, 1.100 ± 0.027), while 4.3% were weak biofilm producers (mean, 0.016 ± 0.002). The OD$_{570}$ results showed that the *Salmonella* serotype influences the biofilm formation capacity on polystyrene ($p < 0.05$). Furthermore, a low PCC (0.121) is found between the *Salmonella* serotype biofilm and multidrug resistance.

| Strains               | Morphotype CRA (n = 91) | Polystyrene Biofilm Formation (n = 91) | OD$_{570}$ * |
|-----------------------|-------------------------|----------------------------------------|--------------|
|                       | rdar pdar bdar saw       | Strong Biofilm Producers               | Weak Biofilm Producers |             |
| *Salmonella Rubislaw* |                         |                                        |               | Mean = 1.083 ± 0.25 |
| (n = 17)              | 6                       | 2                                      | 6            | 3            | 16                                     |
| *Salmonella Newport*  |                         |                                        |               | 1            | Mean = 1.086 ± 0.01 |
| (n = 17)              | 11                      | -                                      | 4            | 2            | 17                                     |
| *Salmonella Infantis* |                         |                                        |               | -            | Mean = 1.085 ± 0.11 |
| (n = 40)              | 24                      | -                                      | 14           | 2            | 40                                     |
| *Salmonella Typhimurium* |                      |                                        |               | -            | Mean = 1.081 ± 0.00 |
| (n = 14)              | 13                      | -                                      | 1            | -            | 14                                     |
| *Salmonella Oranienburg* |                     |                                        |               | -            | Mean = 0.014 ± 0.00 |
| (n = 3)               | -                       |                                       | 3            | -            | 3                                     |

OD$_{570}$ * values ± SDs obtained by polystyrene biofilm formation assays; rdar, red, dry, and rough colony; pdar, pink, dry, and rough colony; bdar, brown, dry, and rough colony; saw, smooth and white colony; OD, optical density; CRA, Congo red agar.

Notably, 59.3% of the *Salmonella* strains (five serotypes) showed the rdar morphotype, 2.1% pdar, 27.4% bdar, and 10.9% saw at both temperatures (22 and 35 °C) in CRA. In the detection of *Salmonella* serotype morphotypes, temperature was not observed to have a significant influence on any of the tested microorganisms ($p > 0.05$). However, there is a medium PCC (0.453) between the *Salmonella* serotype morphotypes and multidrug resistance.

According to the results reported in Tables 1 and 2, four *Salmonella* strain isolates (*Salmonella Rubislaw*-1, *Salmonella Newport*-6, *Salmonella Typhimurium*-8, and *Salmonella Infantis*-9) were selected among all the 91 examined strains based on the obtained biofilm formation values (ODs), multidrug resistance, and morphotype (Table 3). However, *Salmonella Oranienburg* strains (n = 3) are saw and weak biofilm producers.

Additionally, the four *Salmonella* strains selected in this study showed a high ability to develop biofilms in the two culture medias (TSBS and TSBA), reaching more than 7.78 log$_{10}$ CFU/cm$^2$ (Table 4).

In TSBS and TSBA, *Salmonella Infantis*-9 had a significative difference ($p < 0.05$) in the cell density under the tested conditions; however, *Salmonella Newport*-6, *Salmonella Typhimurium*-8, and *Salmonella Rubislaw*-1 did not have differences in their populations ($p > 0.05$). In contrast, in TSBA *Salmonella Rubislaw*-1 had the lowest cell density (7.8 log$_{10}$ CFU/cm$^2$) in comparison to the other serotypes, while *Salmonella Infantis*-9 had the highest cell density (9.6 log$_{10}$ CFU/cm$^2$) in comparison to its population in TSBS. In addition, the observed cells were irreversibly attached, and microcolonies of metabolically active cells were found in the mono-species biofilms of *Salmonella* serotypes on PP, as well as EPS (extracellular polymeric substances) or organic matter from TSBS or TSBA medium by epifluorescence microscopy (Figure 2A–H). SEM analysis of representative mono-species
biofilms showed that cells were linked to each other and embedded in dense EPS or organic matter from TSBS medium (Figure 3A–D).

### Table 3. Characteristics associated with biofilm formation and multidrug resistance of Salmonella serotypes.

| Serotype                  | Resistance Profile      | Phenotypic Characteristics | Morphotype CRA | Polystyrene Biofilm Formation/OD<sub>570</sub> * |
|--------------------------|-------------------------|----------------------------|----------------|-----------------------------------------------|
| Salmonella Rubislaw-1    | AM-CB-GE-NF             | rdar                       | rdar           | SBP Mean = 1.088 ± 0.002                      |
| Salmonella Newport-6     | AMK-CF-CFX-CPF-CL       | rdar                       | rdar           | SBP Mean = 1.094 ± 0.001                      |
| Salmonella Typhimurium-8 | AK-CFX-CPF             | rdar                       | rdar           | SBP Mean = 1.088 ± 0.001                      |
| Salmonella Infantis-9    | AM-CB-CF-NF             | rdar                       | rdar           | SBP Mean = 1.086 ± 0.001                      |

AMK, amikacin; AM, ampicillin; CB, carbenicillin; CF, cephalothin; CFX, cefotaxime; CPF, ciprofloxacin; CL, chloramphenicol; GE, gentamicin; NF, nitrofurantoin; rdar (expresses curli fimbriae and cellulose colonies); SBP, strong biofilm producer; OD<sub>570</sub> * values ± SDs obtained by polystyrene biofilm formation assays.

### Table 4. Cellular density of Salmonella serotypes in mono-species biofilms.

| Serotype                  | Culture Medium | log<sub>10</sub> de CFU/cm<sup>2</sup> ± SD |
|--------------------------|---------------|---------------------------------|
| Salmonella Rubislaw-1    | TSBS          | 8.3 ± 0.51                      |
| Salmonella Newport-6     | TSBS          | 8.2 ± 0.25                      |
| Salmonella Typhimurium-8 | TSBS          | 8.3 ± 0.04                      |
| Salmonella Infantis-9    | TSBS          | 9.6 ± 0.13                      |
| Salmonella Typhimurium ATCC 14028 | TSBS | 9.3 ± 0.10                      |

TSBS, 1/20 diluted tryptic soy broth (TSB) + 1% strawberry residues; TSBA, 1/20 diluted TSB + 1% avocado residues; SD, standard deviation.

**Figure 2.** Epifluorescence microscopy micrographs of mono-species biofilm development on polypropylene type B (PP). Biofilms were developed at 35 °C for 240 h with replacement of culture medium (TSBS or TSBA) at 120 h. Salmonella Infantis-9 on PP in TSBS or TSBA (A,E, respectively), Salmonella Newport-6 on PP in TSBS or TSBA (B,F, respectively), Salmonella Rubislaw-1 on PP in TSBS or TSBS (C,G respectively), and Salmonella Typhimurium-8 on PP in TSBA or TSBS (D,H, respectively). The white bar scale indicates 5 µm.
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Figure 3. Scanning electron microscopy (SEM) micrographs of mono-species biofilm development on polypropylene type B (PP). Biofilms were developed at 35 °C for 240 h with replacement of culture medium (TSBS or TSBA) at 120 h. (A): TSBS organic matter on PP; (B): TSBS organic matter and EPS on PP; (C): biofilms of Salmonella Newport-6 on PP in TSBS; (D): biofilms of Salmonella Rubislaw-1 on PP in TSBS.

4. Discussion

Salmonella Typhimurium, Salmonella Newport, Salmonella Enteritidis, and Salmonella Javiana are some pathogens that were identified as the cause of fresh-produce-related outbreaks of foodborne diseases [2,28]. In addition, Salmonella serotypes have the ability to generate biofilms within the food industry [14,29]. However, certain serotypes have developed resistance to current antibiotics [7,30]. Therefore, understanding the mechanism of resistance and tolerance to antibiotics allows the development of more efficient therapeutic treatments for the removal of a biofilm [31].

In our study, 49.45% of Salmonella serotypes isolated from fruits were multidrug resistant to ampicillin, carbenicillin, amikacin, and ciprofloxacin (Table 1). From previous research, it is evident that Salmonella serotypes have different resistance profiles or antimicrobial susceptibility. Viswanathan and Kaur [32] showed 100% resistance to ampicillin and susceptibility to amikacin, carbenicillin, ciprofloxacin, cefotaxime, and gentamicin in Salmonella spp. isolated from salad vegetables, fruits, and sprouts. Shi et al. [33] showed high (67.5%) multidrug resistance to ciprofloxacin, sulfamethoxazole-trimethoprim, ampicillin, and streptomycin in Salmonella spp. isolated from chicken. However, this study differs in the data for resistance and/or susceptibility to antimicrobials such as amikacin, carbenicillin, ciprofloxacin, cefotaxime, gentamicin, and sulfamethoxazole-trimethoprim. The heterogeneity in antimicrobial resistance patterns observed among Salmonella serotypes could be related to the long-term use of antibiotics as growth promoters in animals, the protection of crops, and poor hygiene practices, which have contributed to antimicrobial resistance [30,34,35].

Additionally, 95.6% of the Salmonella serotypes were strong biofilm producers and 4.39% were weak biofilm producers; however, the Salmonella strains (five serotypes) had differences in their biofilm formation on polystyrene (p < 0.05) in this study. Similarly, other studies have reported high biofilm formation (66.3–67.5%) by Salmonella spp. isolated from humans, animals, or food [5,33]. Amrutha et al. [36] reported that 50% of Salmonella spp. isolated from different fruits and vegetables were strong biofilm producers/ while the rest were found to be moderate or weak biofilm producers. Dhalal et al. [37] showed that S. Heidelberg and Salmonella Typhimurium (turkey isolate) produced less biofilms than Salmonella Typhimurium (chicken isolate) did in 1/10 TSB at 30 °C on polystyrene. Likewise, Tassinari et al. [38] reported significantly greater biofilm formation at 22 °C in comparison to 37 °C by monophasic Salmonella Typhimurium and Salmonella Typhimurium isolated from pig feces, feed, water, and the farm environment, including floors, walls, water drinkers, and troughs. However, Bashir et al. [39] showed that Salmonella serotypes
produced stronger biofilms as the temperature of incubation increased from 10 to 37 °C in 1/20 TSB (p > 0.05).

Consequently, the low correlation (0.121) between Salmonella strains (five serotypes) forming a biofilm and multidrug resistance in our study may be related to the serotype, the source from which strains were isolated (such as water, type of food, farm environment, etc.), the biofilm formation ability, or the excessive use of antibiotics.

In this study, 59.34% of Salmonella strains (five serotypes) exhibited the rdar morphotype, indicating the presence of curli fimbriae and cellulose, extracellular matrix components of the biofilm; however, temperatures of 22 and 35 °C did not cause any differences (p > 0.05).

Similar results were also observed by De Oliveira et al. [6], who reported the rdar morphotype at 28 and 35 °C in 55.2% of Salmonella spp. isolated from raw poultry. In addition, Dev-Kumar et al. [4] and Tassinari et al. [38] reported the rdar morphotype on CRA at 28 °C in monophasic Salmonella Typhimurium, Salmonella Anatum, Salmonella Baildon, Salmonella Braenderup, Salmonella Javiana, Salmonella Montevideo, and Salmonella Newport isolated from water sources, feed, the environment, and feces. In contrast, Trmcic et al. [28] reported that Salmonella Newport isolated from humans and Salmonella Typhimurium and Salmonella Enteritidis isolated from irrigation water exhibited the sar morphotype, while Salmonella Daytona isolated from irrigation water exhibited the bdar morphotype on CRA.

Our results show variations of the morphotypes pdar (2.19%) and bdar (27.47%). This could be associated with the set of genetic machinery behind these morphotypes or with environmental factors such as the nutrients, temperature, pH, and oxygen that regulate the expression of genes involved in the synthesis of cellulose and fimbriae (curli). Römling et al. [40] and Lamas et al. [41] argued that csgD regulates curli fimbria biosynthesis and bcs is responsible for cellulose biosynthesis in biofilm formation by Salmonella.

Consequently, the medium correlation (0.453) between Salmonella serotype morphotypes and multidrug-resistant morphotypes in our study may be related to the extracellular polymeric substances (EPS) of the matrix of the biofilm, such as curli fimbriae, cellulose, extracellular DNA (eDNA), and extracellular polysaccharides that link cells together and confer resistance to antibiotics. In addition, fimbriae and cellulose are coexpressed by Salmonella; a matrix of tightly packed cells produces a spatial organization in which cells in the biofilm cluster in microcolonies [3,42]. Therefore, it is complex to establish an association between multidrug resistance and the formation of a biofilm. In fact, the mechanisms of resistance and tolerance to antibiotics are associated with the bacterial strain and species that generate the biofilm, the formation stage and component of the biofilm (eDNA), antibiotic-degrading enzymes, efflux pumps, and quorum sensing [31,43,44].

All four serotypes of Salmonella used in this study had a high ability to develop biofilms in TSBS (8.39–9.63 log_{10} CFU/cm²) and TSBA (7.81–8.98 log_{10} CFU/cm²) on PP at 35 °C (Table 4). Furthermore, Salmonella Infantis-9 showed a higher cell density that the other serotypes (p < 0.05). Similar results were also observed by Shen et al. [45], who reported a biofilm of Salmonella Newport (mango isolate; 7.2 log_{10} CFU/coupon) and Salmonella Thompson (cilantro outbreak, isolate; 7.5 log_{10} CFU/coupon) cells suspended in 2% lettuce juice extract on stainless steel. Likewise, Singla et al. [46] showed the formation of a biofilm by Salmonella Typhimurium (ST1 and ST2) isolated from vegetables on different surfaces used in the food industry, including PVC (polyvinyl chloride) pipes (ST1 6.2 log_{10} CFU/g; ST2 4.5 log_{10} CFU/g) and polyethylene bags (ST1 5.8 log_{10} CFU/g; ST2 4.2 log_{10} CFU/g); they concluded that PVC pipes are more likely to be colonized by biofilms. De Oliveira et al. [6] reported weak biofilm formation (65.5%) by Salmonella spp. isolated from raw poultry on PVC at 35 °C in BHI (brain heart infusion).

Generally, the development of biofilms by Salmonella serotypes responds to limitation of nutrients through the use of the simplified culture media TSBS and TSBA (p < 0.05). This may be because TSBS has lower nutrients such as amino acids, proteins, and minerals that allow the adhesion and formation of a biofilm; however, TSBA can also contain other
nutrients such as saturated, polyunsaturated, and monounsaturated fatty acids that can influence the formation of a biofilm. Cook et al. [47] reported that biofilm formation by *Salmonella* Typhimurium was higher when grown in lettuce lysates with low sodium than when grown in high-nutrient LB broth. Likewise, the aggregative fimbriae of *Salmonella* spp. are expressed in response to nutrient limitation, under conditions of low osmolarity and low growth temperature, as well as in the stationary phase of growth [48].

Furthermore, in our study, we incorporated TSBA and TSBS in order to simulate the accumulation of organic materials in washing water, as surfaces that come into contact with freshly cut food produce are associated with a poor sanitization process in the commercial fresh produce industry. In fact, food is rich in nutrients and suitable for the growth and reproduction of pathogens; in solid or viscous food, bacteria can easily adhere to the surface of food materials and food processing equipment and can eventually form a biofilm [49]. Hence, operating procedures for cleaning and disinfection must be implemented to prevent the formation or maturation of a biofilm, an accumulation of organic material that can affect the hygienic state of a surface [15].

Our results of epifluorescence microscopy reveal that the biofilm structure is made up of microcolonies of metabolically active cells with the presence of EPS or the organic matter from TSBA or TSBS medium (Figure 2A–F). In addition, acridine orange is intercalated with the RNA, indicating metabolically active cells [24,25]. SEM analysis of biofilms formed by representative mono-species *Salmonella* showed that cells were linked to each other and embedded in dense EPS (Figure 3A,B). However, the components that make up the EPS depend on the microbial species and in particular on their genetics and metabolic pathways, and the concentration of these can vary according to the various carbon or nitrogen sources in the culture medium where the biofilm develops [50]. Therefore, the EPS are the biofilm structure, and these can influence bacterial attachment and transport mechanisms indirectly by impacting both the cell surface hydrophobicity and the surface charge [51].

This can be relevant from a food safety perspective as in this study, the estimation of biofilm formation by *Salmonella* serotype strains shows they have the capacity to form biofilms on PP and polystyrene. Our results agree with studies by other researchers who showed that *Salmonella* is able to form biofilms on polyethylene bags, PVC pipes, plastic surfaces, polystyrene, stainless steel, etc. [16,37,46,52]. Hence, biofilms are reservoirs that may be present on food processing and food contact surfaces, subsequently acting as a potential source of direct and indirect contamination by the introduction of foodborne pathogens into the fresh produce industry.

*Salmonella* serotype biofilms can cause negative impacts in the fresh produce industry, such as reduced operational efficacy in heat exchangers, accelerated metal corrosion, and resistance to the disinfectant used in sanitation procedures. Our results emphasize the importance of incorporating other techniques, such as confocal laser scanning microscopy (CLSM) to determine the components that make up the EPS of biofilms, for implementing strategies for the prevention and removal of biofilms in the fresh produce industry. However, further study is needed to better understand how medium nutrient composition, environmental factors, and strain characteristics influence adherence and biofilm formation by *Salmonella* serotypes.

5. Conclusions

Our study identified the ability of *Salmonella* to form biofilms, depending on the serotype of strains and the environmental conditions. Furthermore, the development of mono-species biofilms by *Salmonella* serotypes respond to nutrient limitation with the use of simplified culture media such as TSBA and TSBS. However, more studies are needed to investigate the interactions between strains and components of the biofilm matrix to devise effective interventions and strategies for preventing and removing biofilm to decrease the contamination of fresh products.
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