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Evaluation of *Salmonella* biofilm attachment and hydrophobicity characteristics on food contact surfaces

Colton Ivers[1], Eda C. Kaya[1], Umut Yucel[1], Dan Boyle[2] and Valentina Trinetta[1*]

Abstract

Salmonella forms biofilms, and persist on food contact surfaces. Once a biofilm is formed cleaning and sanitation protocols may be inadequate for effective removal. This study evaluated attachment characteristics, surface properties, and structure of *Salmonella* biofilms on food contact surfaces commonly used in the tree-fruit industry. Multi-strain *Salmonella* biofilms were grown in a Centers for Disease Control and Prevention (CDC) biofilm reactor at 22 ± 2 °C and sampling was conducted at 2, 24 and 96-h. After each incubation period, coupons were gently rinsed and the remaining cells enumerated. Biofilms were analyzed with Laser Scanning Confocal Microscopy (LSCM). Hydrophobicity was evaluated by measuring the contact angles of reference liquids method using a drop tensiometer instrument. Material type and biofilm age significantly influenced attachment and biofilm hydrophobicity ($P < 0.05$). The strength of attachment, across all time points, was highest on nylon followed by wood and high-density polyethylene. The highest contact angle measurements were observed after 96-h of biofilm formation for each material. All the results and observations from this study contribute to a better understanding of the attachment and hydrophobicity characteristics of *Salmonella* and might help producers make informed decisions when selecting containers for harvesting and storing in order to minimize biofilm formation and potential for cross-contamination.

Keywords *Salmonella*, Biofilm, Hydrophobicity, Attachment

Impact statement

This study highlights the role of biofilm formation and the influence of hydrophobicity properties of both biofilms and surface materials. Results showed that biofilm characteristics are influenced by surface type and incubation time, underscoring the importance of making informed decisions when selecting containers for harvesting and storing produce in order to minimize biofilm and potential for cross-contamination.

*Correspondence:
Valentina Trinetta
vtrinetta@ksu.edu
[1] Food Nutrition Dietetics and Health Department, Kansas State University, 1310 Research Drive Park, Manhattan, KS 66502, United States
[2] Division of Biology, Kansas State University, 6 Ackert Hall, Manhattan, KS 66506, USA

Background

Salmonella foodborne infections are a major public health concern and account for an annual economic burden of \$4.1 billion in the United States [1]. *Salmonella* causes more hospitalizations and deaths than any other foodborne pathogen. Fruits and vegetables are often linked to over 25% of *Salmonella* outbreaks [2]. Produce can be contaminated though multiple sources during harvesting and processing. When harvesting equipment is not effectively cleaned and sanitized, it may serve as a reservoir for *Salmonella* biofilm formation and potentially cross-contaminate fresh produce. Preventing contamination during harvest is vital for produce safety. *Salmonella* has been shown to persist on packing line materials for over 28 days under environmental conditions consistent with Florida's fall/winter tomato

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production season) [3]. The same research also reported that surfaces such as stainless steel, PVC, and unfinished oak wood could support the survival of *Salmonella* over an extended period of time. *Salmonella* can attach to various surfaces in the food industry and form biofilms [3].. A biofilm is defined as a group of organized cells attached to a surface and bound together by the Extracellular Polymeric Substances (EPS) [4]. The life cycle of biofilms is generally viewed as cyclical and consists of five stages: initial attachment, irreversible attachment, development, maturation, and dispersion [5].). Initial bacterial attachment can be highly influenced by the physical and chemical properties of the environment and bacterial cell. Environmental factors that influence bacterial attachment may include shear force, surface and cell hydrophobicity, surface conditioning and roughness, pH, and nutrient levels [4].. In this initial stage bacterial cells can easily detach or be removed from the surface. Irreversible attachment occurs when cells begin to form a matrix primarily composed of EPS [5]. Moreover, the EPS matrix provides necessary structure to stabilize the biofilm and interact with the surface to form a robust connection [5]. At this point cells are firmly attached to the surface and cannot be easily removed.

Salmonella biofilm formation is a major food safety issue as it may increase pathogen persistence in processing environments [6], contaminate products, and resist routine cleaning protocols [3].

The ability of bacteria to adhere to a surface is a complex process and influenced by the chemistry of the surface and its interactions with bacteria [7]. Surface hydrophobicity (measured by contact angle) is one of the critical parameters that affect bacterial attachment, and biofilm formation [7]. However, there are contradictory findings in the literature regarding the relationship between surface hydrophobicity and bacterial attachment [8, 9]. This is partly because other surface characteristics, such as porosity and surface topography, which are also known to affect hydrophobic behavior, can affect bacterial attachment. Furthermore, many researchers agree that cellular attachment is highly complex and influenced by several other factors such as serovar, material, nutrient, temperature, and pH [10–12].

Harvesting equipment such as nylon picking bags and wooden and high-density polyethylene (HDPE) harvest bins are commonly used in the tree-fruit industry. The prevalence of *Salmonella* [13] and indicator organisms [14] on produce processing equipment coupled with pathogen's potential to form strong biofilms [15, 16] imply that these surfaces may serve as reservoirs and could cross contaminate produce [3]. Therefore, this study aims to evaluate the formation, attachment and hydrophobicity characteristics of *Salmonella* biofilms grown on nylon, wood, and HDPE.

Methods

Bacterial strains
Three *Salmonella* serovars were used in this research. *Salmonella* Enteritidis ATCC BAA-1045 isolated from raw almonds, *S.* Agona LJH 517 from alfalfa sprouts, and *S.* Newport ATCC 6962 from a clinical isolate. Each strain was preserved in CryoCare Bacteria Preservers system (Scientific, Stamford, TX). The day before the experiment one bead for each serovars was removed from the ultra low freezer and transferred in 10 ml of freshly made Tryptic Soy Broth (TSB, BD Difco, Sparks, MD) and incubated for 24-h at 37 °C with 70 rpm shaking. The overnight cultures were streaked on Tryptic Soy Agar (TSA, BD Difco, Sparks, MD) to confirm purity. A single colony from each TSA plate was incubate for 24-h at the conditions described aboved. Equal amount from each bacteria suspension (1 ml) were combined to obtained a final bacterial cocktail population of 10^9 CFU/ml [17]. Each serovar solution was also enumerated (10^7 CFU/ml).

Biofilm growth
Biofilms were grown in a Center for Disease Control and Prevention (CDC) biofilm reactor (Biosurface Technologies, Bozeman, Montana) for up to 96-h using a protocol developed in our lab [17]. Briefly, during the batch phase, 1 ml of of *Salmonella* cocktail was added to TSB at a concentration of 3 g/l to obtain a final concentration in the reactor of 10^3 CFU/ml of bacteria. After 24 h, the continue phase started and fresh media (at a concentration of 1.5 g/l) was pumped at a rate of 7 ml/min for 72-h. The reactor was maintained at 22 ± 2 °C and the stir plate set at 120 rpm.

Coupon material
The following materials were selected to represent picking bags and harvest bins commonly used in the tree-fruit industry: nylon, wood, and high-density polyethylene (HDPE). All coupons had 1.27 cm diameter on both sides for biofilm growth. Nylon material was cut from used apple harvest picking bags. Sheets of basswood were purchased from a local store and cut into 1.27 cm diameter circles with a Glowforge Pro laser cutter (Glowforage, WA). HDPE coupons were purchased from Biosurface Technologies Corporation (Bozeman, MT). All coupons were scrubbed with soapy water, sonicated for five-minute, and rinsed with tap water. This process was repeated a total of three times. Clean coupons were placed under UV light for 30 min before transferred in the biofilm reactor. The

fully assembled reactor was then autoclaved at 121 °C for 15-min.

Biofilm attachment

Salmonella biofilms were grown as described above and at selected time intervals (2, 24 and 96 h), a coupon for each material was removed for biofilm attachment analysis. Inoculated but not rinsed coupons were considered positive controls, while un-inoculated coupons placed in TSB for 96-h served as negative controls. Coupons were gently rinsed by placing them in 50 ml of sterilized deionized water for 10 s [18]. Next, coupons were transferred in a new beaker for 10-s, followed by a third rinse to remove loosely attached cells. Afterwards, coupons were allowed to dry for 30-min prior to enumeration and then placed in 10 ml of Phosphate-Buffered Saline solutions (PBS, VWR, Solon, OH). Biofilms were detached from coupons following the ASTM method E2871-19 [19]. Serial dilutions were performed in 0.1% peptone water (Bacto, Sparks, MD) and spread plated in duplicate on Tryptic Soy Agar (TSA, BD Difco, Sparks, MD). Colonies were counted and reported as log CFU/cm^2 after 18–24 h of incubation at 37 °C. Two compouns for each conditions and time interval were used during each trial. Experiments were performed in triplicate.

Hydrophobicity assay

Hydrophobicity experiments were conducted using an optical drop tensiometer (Attention Theta Flex C311, Nanoscience Instruments, Phoenix, Arizona) using *Salmonella enterica* serovar Typhimurium LT2 biofilms. This strain was used as a BSL 1 surrogate for *S.* Enteritidis ATCC BAA-1045, *S.* Agona LJH 517, and *S.* Newport ATCC 6962 since experiments were conducted in a BSL 1 laboratory. Clean not-inoculated coupons and clean inoculated coupons soaked in TSB for 96-h were used as controls for comparison purposes. The hydrophobicity of *Salmonella* LT2 biofilms grown on nylon, wood, and HDPE were measured at selected time intervals (2, 24, and 96-h) using sessile-drop technique [18].. All coupons were allowed to dry for 1-h prior to assay. Aliquots (10 µL) of reference liquids, deionized water and formamide, were placed on the surfaces and the contact angles were automatically captured by optical drop tensiometer, calibrated by using a steel sphere with known dimension (35 mm), over a 30-s period. Measurements were taken in quadruplicate and at two different locations on each coupon. Two compouns for each conditions and time interval were used during each trial.

Laser scanning confocal microscopy (LSCM)

Salmonella cocktail biofilms grown on nylon, wood, and HDPE were imaged under a Laser Scanning Confocal Microscopy (LSCM) (LSM-5 Pascal associated with a Zeiss Axioplan 2) at the Microscopy Facility of the Biology Department at Kansas State University. Samples were imaged at 2, 24, and 96-h. The samples were stained with SYTO 9 (ThermoFisher Scientific, Eugene, OR) and SYTOX red (ThermoFisher Scientific, Eugene, OR) to differentiate live and dead cells [18]. Images and 3D projections were developed using ImageJ (NIH, USA).

Statistical analysis

Coupons were randomly assigned treatments across experiments. The attachment assay was performed in triplicate for each time condition tested (2, 24 and 96-h) and material (nylon, wood and HDPE). Coupons inoculated with Salmonella cocktail but not rinsed were considered positive controls, while not-inoculated coupons in TSB for 96-h as negative controls. Two coupons for each treatment were used during the indipendednt trials. The hydrophoboicity assay was instead performed in triplicate. Also for this experiment two coupons for each time condition tested (2, 24 and 96-h) and material (nylon, wood and HDPE). Clean not-inoculated coupons and clean inoculated coupons soaked in TSB for 96-h were used as controls for comparison purposes. *Salmonella* enterica LT2 was used to form biofilm, since assays were permorned in a BSL1 laboratory. Statistical differences were determined at $P < 0.05$. All results were analyzed in SAS Studio (Cary, NC) using the General Linear Model (GLM) procedure with Tukey's multiple comparison.

Results and discussion
Biofilm attachment

It is important to understand *Salmonella* attachment and biofilm formation to help control pathogen contamination on food contact surfaces commonly used in the food industry. In this study we evaluate *Salmonella* biofilm formation on materials commonly used in the produce industry: wood, nylon and HDPE. Incubation time and material were found to be statistical significant for *Salmonella* biofilm attachment assay ($P < 0.05$). Base on the statistical analysis the interaction between incubation time and material was also significant ($P < 0.05$), indicating that the growth of *Salmonella* is influenced was positively influenzed by time, but that the type of material where the biofilm forms impacted attachment as well. Observations revealed that. *Salmonella* attachment was greatest on nylon followed by wood and HDPE ($P < 0.05$) and increased over time (2-, 24-, and 96-h), regardless

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the material used ($P < 0.05$). Control coupons with 96-h biofilms and no rinse step resulted in *Salmonella* populations of 10.00 ± 0.07, 9.72 ± 0.14, and 8.86 ± 0.19 Log CFU/cm^2 on nylon, wood, and HDPE, respectively. No microbes were detected on non-inoculated coupons soaked in TSB for 96-h (negative controls). *Salmonella* population in respect to incubation time and material is presented in Fig. 1. The 2-h incubation period resulted in different *Salmonella* populations on nylon, wood, and HDPE: 7.69 ± 0.25, 6.66 ± 0.43, and 4.73 ± 0.23 Log CFU/cm^2, respectively ($P < 0.05$). When incubation time increased from 2-h to 24-h, *Salmonella* attachment increased significantly on each coupon: 9.43 ± 0.02, 8.41 ± 0.59, and 7.06 ± 0.28 Log CFU/cm^2 on nylon, wood, and HDPE, respectively ($P < 0.05$). Nevertheless, the populations recovered from 24-h HDPE were comparable to those of 2-h nylon and wood ($P > 0.05$); similar populations were also observed between 24-h wood and 2-h nylon ($P > 0.05$). *Salmonella* populations on wood and HDPE rose further when incubated for 96-h; 9.68 ± 0.08 and 8.59 ± 0.10 Log CFU/cm^2, respectively ($P < 0.05$); however, nylon only increased slightly to 10.03 ± 0.06 Log CFU/cm^2 ($P > 0.05$). Attachment after 96-h on nylon and wood was similar to 24-h nylon, while 96-h HDPE and 24-wood exhibited a similar degree of attachment. Similarly to previous research, attachment was significantly impacted by surface type [9, 20, 21]. and incubation time [18, 22]. *Salmonella* isolates from poultry sources

attached greater on polycarbonate surfaces compared to stainless steel [23].Another study) [24] found that the initial surface coverage of *Salmonella* was dependent on stainless steel finish and increased over a 5-day incubation period. In the current study, we also observed that *Salmonella* attachment increased over time.

Hydrophobic properties of surfaces and biofilms

Deionized water and formamide were used as reference liquids to elucidate the physiochemical characteristics of biofilms (Table 1). Previous researchers have suggested that pathogen attachment is favored on hydrophobic surfaces [21]. *Salmonella* attachment and material hydrophobicity was positively correlated between select materials but they were not able to correlate attachment and hydrophobicity of all materials [9]. In the present study, we used a BSL1 *S. enterica* serovar Typhimurium LT2 to form biofilm and we compared the results to clean not-inoculated coupons and clean inoculated coupons soaked in TSB for 96-h. In this study, we observed that solvent type significantly influenced contact angle values ($P < 0.05$): formamide resulted in lower contact angle values than water. A significant different in contact angle values between formamide and deionized water was observed because of their difference in polarity and absorptivity [18]. Statistical analysis revealed that material type and incubation time were statistical significant in this assay ($P < 0.05$). The value of contact angles
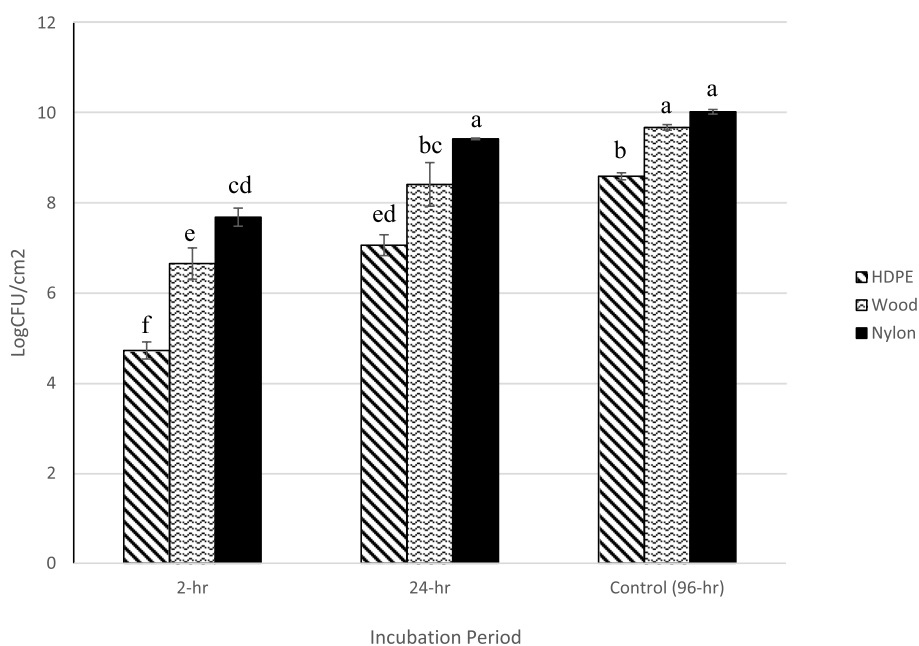
Fig. 1 *Salmonella* (Log CFU/cm^2) remaining population on nylon, wood, and HDPE after 2, 24, and 96-h incubation and rinse step. The following counts were recovered from mature 96-h biofilm with no rinse step; 10.00 ± 0.07, 9.72 ± 0.14, and 8.86 ± 0.19 CFU/cm^2 on nylon, wood, and HDPE, respectively. Small letters indicate statistical differences between treatments

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Table 1 Contact angle measurements of biofilms grown on wood, nylon, and HDPE coupons using two different solvents (A) water and (B) formamide. Control 1 represents contact angle values measured on dry coupons with no cells. Control 2 represents contact angle values measured after materials were soaked in TSB for 96-h without inoculum. Small letters and Roman numerals indicate values with statistical difference (P < 0.05) for each column and row, respectively

		Contact angle [°]		
Solvent	Conditions	Nylon [°]	Wood [°]	HDPE [°]
Water	Control 1	27.55 ± 1.13[ab,I]	34.53 ± 0.45[b,II]	55.29 ± 0.57[a,III]
	Control 2	30.13 ± 0.78[c,I]	34.83 ± 0.21[b,II]	61.93 ± 0.43[e,III]
	2 h	33.99 ± 0.16[de,I]	36.20 ± 0.29[b,II]	64.62 ± 0.64[f,III]
	24 h	35.85 ± 0.21[e,I]	42.25 ± 0.39[e,II]	66.96 ± 0.66[g,III]
	96 h	40.83 ± 0.98[f,I]	47.82 ± 0.74[f,II]	71.10 ± 0.98[h,III]
Formamide	Control 1	25.89 ± 1.39[a,I]	32.50 ± 0.38[a,II]	52.42 ± 0.59[b,III]
	Control 2	28.16 ± 0.26[b,I]	32.42 ± 0.38[a,II]	57.33 ± 0.55[c,III]
	2 h	30.35 ± 0.29[c,I]	34.93 ± 0.13[b,II]	59.56 ± 0.37[d,III]
	24 h	32.73 ± 0.41[d,I]	39.44 ± 0.4[c,II]	59.74 ± 1.18[d,III]
	96 h	38.56 ± 0.57[g,I]	44.00 ± 1.24[e,II]	67.95 ± 0.71[g,III]

increased over time for all the materials analyzed, while overall wood was the least hydrophobic surface followed by nylon, while HDPE was the most hydrophobic surface, indicating the ability of *Salmonella* biofilm to form a bigger contact angle (less surface tension) with HDPE as compared to wood. Biofilm on HDPE will be less attached to the surface as compared to the ones grown on wood.

Material type and biofilm maturity significantly affected contact angles (*P* < 0.05). All materials evaluated presented contact angles < 90° characteristic to hydrophilic surfaces [25]. The contact angle measurements obtained from clean, uninoculated, coupons (controls) indicate HDPE as the most hydrophobic surface (55.29 ± 0.57°) followed by wood (34.56 ± 0.44°) and then nylon (27.55 ± 1.21°). When soaked in sterile TSB for 96-h contact angle increased on nylon and HDPE (*P* < 0.05), while the contact angle observed on wood increased slightly (*P* > 0.05). Nevertheless, clean and TSB control coupons resulted in the lowest contact angles. Biofilms at 2-h on nylon and HDPE significantly increased contact angle (*P* < 0.05); however, the contact angle observed on wood remained similar to the controls (*P* > 0.05). Contact angle values further increased with 24-h- biofilms, indicating the increase in hydrophobic surface properties. The trend of hydrophobicity continued as 96-h biofilms formed on nylon, wood, and HDPE resulted in the highest contact angle value (*P* < 0.05). No correlation between *Salmonella* attachment and hydrophobicity of AISI 304 and 316 stainless steel was observed in a recent study [8].

The differences in hydrophobicity between clean surfaces (i.e., without biofilm, control) need to be evaluated considering not only the chemical nature of the materials but also their surface topology. The porosity on the surface, for example, can increase hydrophilic behavior via capillary effects. Similarly, textural surface characteristics are known to alter the surface hydrophobicity to varying extents.

Cell surface hydrophobicity plays a key role in biofilm formation and resistance to biocides. Since biofilm formation is thought to be a strategy for survival under a variety of environmental stress, it seems that hydrobobicity might be a relevant physico-chemical factor that relates to biofilms yield and resistance to antimicrobials. Nevertheless, this observation should be further investigated.

Laser scanning confocal microscopy (LSCM)

The intensity of fluorescently stained *Salmonella* cells was measured by Laser Scanning Confocal Microscopy (LSCM) and subsequently rendered into 3D models to visualize biofilm development (Fig. 2). The 3D models complement the quantitative data, where we observed that *Salmonella* biofilm attachment was influenced by incubation time and material with stronger attachment on wood and nylon as compared to HDPE (in Fig. 1) and provide insight on the attachment and aggregation characteristics of *Salmonella* biofilms over a 96-h period. Biofilms grown on nylon coupons displayed a dense accumulation of cells after 2-h of formation (Fig. 2A). Further aggregation of cells was observed after 24 and 96-h. Wood has been shown to autofluorescence under LSCM [26]. Similarly, we observed slight autofluorescence of wood at 0-h (Fig. 2B). Nevertheless, biofilms grown on wood initially appeared to form smaller colonies dispersed across the coupon and then exhibited increased surface coverage as biofilm maturity increased. Conversely, the initial biofilm formation on HDPE resulted in the least fluorescence (Fig. 2C). After 24-h aggregates of cells were dispersed widely across the materials and surface coverage was greatest after 96-h of biofilm formation. Overall, these models further show the physical and physiochemical properties of nylon and wood are more favorable to *Salmonella* attachment than HDPE. In our study, LSCM analysis showed that nylon surface has uneven nylon fibers that lead to a porous structure to further alter its hydrophobic characteristics. In comparison, the surface of HDPE films was smooth with limited porosity to reflect the chemical nature of the material. Therefore, the attachment was the lowest for the HDPE coupons. Similarly, another research [27], where CDC biofilm reactor was used to evaluate bacteria adhesion, observed that surface roughness enhanced attachment

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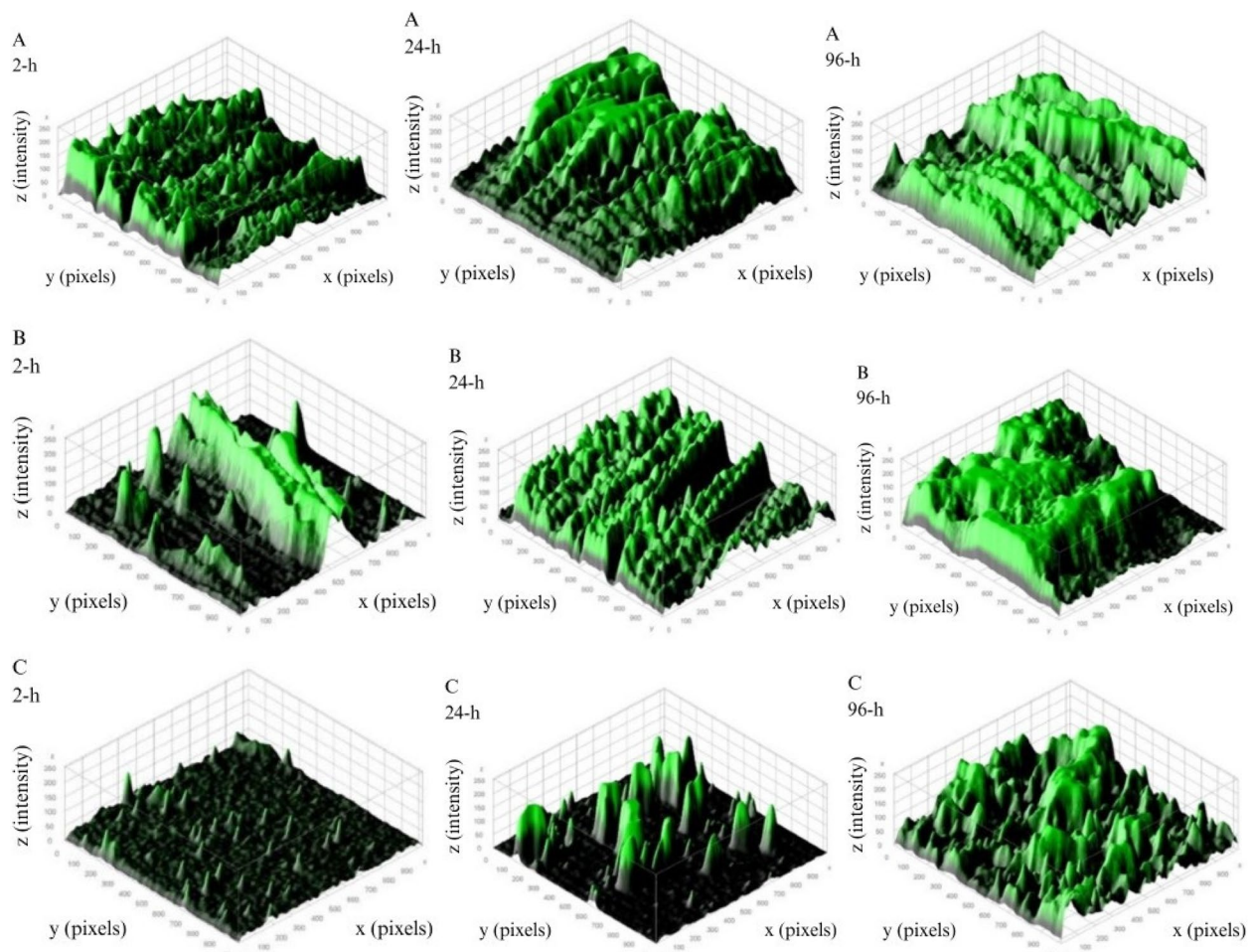
Fig. 2 3D surface projections of *Salmonella* biofilms on (**A**) nylon, (**B**) wood, and (**C**) HDPE after 2, 24, and 96-h of growth in CDC Biofilm Reactor. Samples were imaged with Laser Scanning Confocal Microscopy (LSCM). Green represents living *Salmonella* cells. Z-axis represents fluorescent intensity; X and Y-axis represent pixels. All images were processed with ImageJ

and biofilm formation. In our study a higher attachment was reported for wood and nylon, where increase surface area and niche formation may have favored attachment for bacteria [28, 29]. LSCM was also utilized to quantify *Salmonella* biofilm depth and surface coverage on various stainless-steel surfaces [24]. Authorsobserved that biofilms formed after 1-day on electropolished stainless steel exhibited significantly less surface coverage and similar depth as compared to non-treated stainless-steel surfaces. Interestingly, after 5-days, biofilm surface coverage and depth increased dramatically on both surface types. However, non-treated stainless steel presented significantly greater surface coverage and biofilm depth. These observations further demonstrate the impact of surface type and time on biofilm formation characteristics. In the current study, we observed that 2-h biofilms on wood and HDPE formed dense, widely dispersed colonies and increased surface coverage with extended incubation times. Whereas, 2-h biofilms grown on nylon displayed complete surface coverage and increased overall biofilm mass with extended incubation times. HDPE resulted in the least colonization of *Salmonella* across all time periods. This is not surprising as it was the only hard non-porous food contact surface evaluated and it is typically regarded as an acceptable surface in terms of hygienic design and cleanability.

Conclusion

The results collected in this study should be considered by the tree-fruit growers when selecting materials used for harvesting bins and bags. Overall, *Salmonella* attachment and biofilm characteristics were significantly impacted by surface type and incubation time. This research suggests that alternative materials should be considered in place of wood and nylon, since they can favor microbial niche and biofilm formation.

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Availability of data and materials

Data available on request to Valentina Trinetta (vtrinetta@ksu.edu).

Declarations

Ethics approval and consent to partecipate

Not applicable.

Consent for publication

Not applicable.

Competing interests

The authors declare no competing interests.

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