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Interaction of *Salmonella* with *E. coli* and *Proteus* spp. in Biofilm Formation

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Authors' contributions

This work was carried out in collaboration among all authors. All authors read and approved the final manuscript.

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Original Research Article

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ABSTRACT

Aims: Investigate the interaction of *Salmonella* spp. with *E. coli* and *Proteus* spp. in biofilm formation as mono and dual-species at different time durations

Experimental Design: *Salmonella*, *Proteus,* and *E. coli* were isolated from Broiler chicken meat, and the biofilm-forming ability of these organisms were studied.

Place and Duration of Study: The study was conducted at the Laboratory of Livestock Production, Faculty of Agricultural Sciences, Sabaragamuwa University of Sri Lanka, from 2019 December to 2020 May.

Methodology: This study investigated the biofilm-forming ability of *Salmonella* as a mono species and its interaction with *E. coli* and *Proteus* in the process of biofilm formation. Microorganisms used for this study were isolated from broiler chicken meat. Biofilm was quantified using a microtitre plate assay. The interaction effects were tested at the temperature of 28^0C in different time durations (up to 120 hours).

Results: *Salmonella* 1 and *Proteus* monocultures showed significantly higher biofilm-forming ability

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than *Salmonella* 3 isolate at all tested time points. At 120 hr, additionally to the *salmonella* 1 and *Proteus* isolates *E. coli* also formed significantly higher biofilms than *Salmonella* 3. However, *Salmonella* 3 was the lowest biofilm former as mono biofilm at all tested time durations. *Salmonella* 1 interaction with *Salmonella* 3 isolates formed less biofilms than *Salmonella* 1 mono biofilm at 48hr and 72hr correspondingly. Salmonella 1 and its interactions with *Salmonella* 3, *Proteus*, *E. coli* showed similar biofilm-forming abilities without significant differences at all other tested time points. Specifically, *Salmonella* 3 interaction with *Salmonella* 1 as dual biofilm showed higher biofilm-forming ability than *Salmonella* 3 mono biofilm at all tested time points. Tested isolates and their interaction achieved the highest biofilm formation at numerous time points. In fact, at 48hr, *Salmonella* 3 isolates and its interaction of *Proteus*, *E. coli,* and *Salmonella* 1 interaction with *Proteus* attained their highest biofilm formation abilities. The highest biofilm formation was achieved by *Salmonella* 1 isolate as mono biofilm and *Salmonella* 1 interaction with *E. coli* as dual biofilm at 72hr. Biofilm-forming trend of respective isolates and interactions showed numerous patterns at tested time durations.

Specifically, *E. coli* rapidly enhanced its biofilm-forming ability as monoculture from 24 hr to 120 hr. *Proteus*, *Salmonella* 3 as monocultures, *Salmonella* 3 interaction with *Proteus* and *E. coli* as dual cultures showed progressive biofilm development from 24 hr to 48 hr. *Salmonella* 1 monoculture and its interaction with *Salmonella 3*, *E. coli* as dual biofilm improved their biofilm-forming ability from 24 hr to 72 hr. Similar to *Salmonella* 3 interaction with *Proteus*, *Salmonella* 1 interaction with *Proteus* also increased its biofilm-forming ability from 24 hr to 48 hr.

Conclusions: This study concluded that there is a variation among isolates and their combinations in forming the biofilms, where there is an enhancement of biofilm in dual-species over the mono-species in some interaction, and there is a reduction in biofilm formation by dual-species with some combinations. Further, this concluded that Salmonella is interacting with other commonly found bacteria such as *Proteus* and *E. coli* in biofilm formation.

Keywords: Dual biofilm; E. coli; interaction; mono biofilm; proteus; quantification; Salmonella.

1. INTRODUCTION

Foodborne diseases resulting from consuming contaminated food have become a major problem that puts human health at a greater risk. According to world statistics, unsafe food consumption causes 420,000 global deaths annually [1]. *Campylobacter, Salmonella, Listeria* and *Escherichia coli* are the most significant pathogenic bacteria posing severe foodborne outbreaks globally [2]. *Salmonella* is associated with contamination of a wide range of foodstuffs such as meat, shrimps, vegetables, fruits, etc. [2], which ultimately leads to food safety issues. *Salmonella* is a Gram-negative bacterium, leading to typhoidal, paratyphoid fever, and non-typhoidal salmonellosis. In fact, *Salmonella* Typhi is the major causative agent for typhoid fever while *Salmonella* Paratyphi is the causative agent for paratyphoid fever, beyond that two; other serovars generates non-typhoidal salmonellosis. Enteric fever is the main symptom of typhoidal and paratyphoid fever, while non-typhoidal salmonellosis is characterized by gastroenteritis. Despite the foodborne nature, animals are the major reservoir of non-typhoidal salmonellosis [3]. *Salmonella* and *E. coli* bacterial strains, such as Shiga-toxin-producing strains (STEC) and enterotoxigenic E. coli (ETEC) strains, pose negative health impacts on humans, causing foodborne illness. ETEC causes traveler's diarrhea, while STEC causes bloody diarrhea and abdominal cramps with or without mild fever [4]. *Proteus,* a gram-negative facultatively anaerobic, heterotrophic, and proteolytic rods frequently associated with urinary tract infections, also speculated their potentially harmful effect of gastroenteritis in humans [5,6,7].

These bacteria live in different environments, and for survival in various conditions, they use several survival mechanisms. Biofilm formation is one kind of survival mechanism used by bacterial communities in different environments, such as food-related environments. Biofilms are the mono or multi-species (mixed) bacterial communities attached to biotic or abiotic surfaces with enmeshed extracellular matrix [4,8]. *Salmonella* is one of the biofilm-forming bacteria, exists in highly nutritive broiler meat surfaces and related surfaces, either as mono biofilms or multi-species biofilms [9,10,11], which ultimately leads to cross-contamination and foodborne illness. Apart from foodborne illness, biofilms formation has become a great dilemma as it poses additional negative impacts such as antibiotic/disinfectant resistance and metal corrosion. *Salmonella* mono biofilms resist commonly used antibiotics such as ciprofloxacin, azithromycin, cefotaxime, tetracycline, and penicillin. The resistance is affected by inhibitory activities mediated by efflux pumps with existing drug resistance gene profile, presence of extracellular matrix, and slow growth rate achieved by biofilms under stress conditions [12,13]. However, some studies described that the *Salmonella* multi-biofilms are resistant to their mono biofilm status [14,15,16,17]. This enhancing resistance may be due to the chemical interaction of different polymers produced by multi-species bacteria, specific bacterial arrangement patterns, competitive interaction, quorum sensing behavior, and horizontal gene transfer [18].Only very few literature on *Salmonella* interaction with other bacterial species in biofilm formation and the sensitivity of biofilm cells to commonly used disinfectant agents. Due to the paucity of available literature, this study was conducted to investigate the interaction of *Salmonella* spp. with *E. coli* and *Proteu*s spp. in biofilm formation as mono and dual-species at different times durations.

2. METHODOLOGY

This study investigated the biofilm-forming ability of *Salmonella* as a mono species and its interaction with *E. coli* and *Proteus* in the process of biofilm formation. Microorganisms used for this study were isolated from broiler chicken meat. Biofilm was quantified using microtiter plate assay. The interaction effects were tested at the temperature of 28^{0}C in different time durations (up to 120 hours).

2.1 Sample Collection

Fifty broiler chicken meat samples collected from retailer broiler meat shops located at Rathnapura district, Sri Lanka were used in this study. All the samples were transported under the chill condition to the Laboratory of Livestock Production, Faculty of Agricultural Sciences, Sabaragamuwa University of Sri Lanka. On arrival, sample processing was started without any delay.

2.2 Isolation of *Salmonella*, *E.coli* and *Proteus* Species from Broiler Chicken Meat

Isolation of *Salmonella*, *E. coli* and *Proteus* was done as the method described in the guidelines of FDA manual [10,19] briefly twenty five gram portion of the each broiler meat sample was aseptically removed and homogenized with 225 ml lactose broth (HiMedia Laboratories, India) for 2 minutes. The homogenized mixture was incubated for 24 hours at 37^{o}C for completing the pre-enrichment step. As the next step, selective enrichment was done in three types of broths (selenite cystine broth (SCB) and tetrathionate broth (TTB), and Rappaport-Vassiliadis broth (RVB)). In the selective enrichment, one milliliter of each pre-enriched sample were added to 10 ml each of SCB and TTB (HiMedia Laboratories, India). In contrast, 0.1 ml was added to 10 ml of RVB. The inoculated SCB and TTB were incubated at 37^{o}C for 24 hours, while RV broth was incubated at 43^{o}C in a water bath for 24 hours. Apart from that, 1 mL of each pre-enriched sample was also inoculated to MacConkey broth (H iMedia Laboratories, India) to enrich the E. coli species and allow overnight incubation at 37^{0}C. Then selective plating was done in Hektoen Enteric Agar (HEA), bismuth sulphite (BSA) agar and xylose-lysine-desoxycholate agar (XLD) (Hi Media Laboratories, India) for isolating *Salmonella* and *Proteus*. At the same time, MacConkey agar (Hi Media, India) isolated *E. coli* species. Loop full each from these broths were streaked on Hektoen Enteric Agar (HEA), bismuth sulphite (BSA) agar, and xylose-lysine-desoxycholate agar (XLD) and incubated at 37^{o}C for 24 hours. For *E. coli* isolation, full loop culture from each sample was streaked on macConkey agar plates and incubated 24hr, 37^{0}C, and subculturing was done until pure colonies were obtained. Five pure, presumptive colonies from each selective plate were subjected to a battery of biochemical tests such as sugar fermentation, indole production test, urease production, and MR- VP test, citrate utilization test done for distinguishing the *Salmonella, E. coli,* and *Proteus* species. In fact, *Salmonella* and *Proteus* were differentiated using the urease production test, and *E.coli* was distinguished by using the indole production test.

2.3 Quantification of Biofilm Formation by *Salmonella* as Mono spp. and its Interaction with E*. coli* and *Proteus* spp

After the isolation procedure, three bacterial isolates (*Salmonella* spp., *Proteus* spp., and *E.coli*) and their combinations (Table 1) were used to investigate the biofilm-forming ability of *Salmonella* as mono biofilm and *Salmonella* interaction with *Salmonella*, other spp. Such as

E. coli and *Proteus* as dual biofilms. Descriptively two *Salmonella* isolates (SAL 1 and SAL 2), one *E. coli* isolate and one *Proteus* isolate were used in this study as below mentioned in Table 1.

Bacterial cultures were grown in 96 well microtiter plates (Grenier Bio-one, Germany) as mono and dual cultures in triplicates, as indicated in Fig. 1. Cultures were inoculated at 10^5CFU /ml to Luria-Bertani broth (Hi media, India) in microtiter plates and subsequently incubated at 28ºC for different time intervals (24, 48, 72, 96 and 120 hours), allowing them to form biofilm on the microtitre plate. At every time point, biofilm formation was qualified using the method described by Stepanovic et al. [20] and with the modification described by Jayaweera et al. [11].

Experiments were carried out in triplicates, and uninoculated negative control was maintained. Quantification of biofilms was done by staining the biofilms with crystal violet at the end of each incubation, as described by Stepanovic et al. [20]. Briefly, the contents of the wells were aspirated and washed thrice with sterile phosphate-buffered saline (PBS) (pH – 7.2) as the amount of 250 µl per well in each washing. The plates were vigorously shaken to remove all unattached planktonic cells. The remaining attached bacterial cells were fixed with 200µl of methanol for 15 minutes, and wells were emptied and air-dried. Afterward, the staining was done with 2% crystal violet for five minutes to stain the biofilms on the microtiter plates. After the process, the excess stain was removed, and the plates were washed properly and rinsed by gently submerging the plates in a water tub with gentle shaking. Then the plates were allowed to dry for another 15 minutes. After drying the stained biofilm cells, the stained biofilm cells were resolubilized with 230µl of 33% (v/v) glacial acetic acid. Following resolubilizing, the cells, optical density [20], was measured at 600nm wavelength by spectrophotometer (Multiskan sky with touch screen Microplate Spectrophotometer, Thermo Fisher Scientific., Waltham, MA USA).

Table 1. Bacterial isolates and combinations used in this study

Bacterial species and combination of bacterial spp.	Codes
Salmonella spp.	SAL 1
	SAL 3
Proteus spp.	P
E. coli spp.	E
Salmonella spp.+ *Salmonella* spp.	SAL1+SAL3
Salmonella spp. + *Proteus* spp.	SAL 1+P
	SAL 3+P
Salmonella spp.+ *E. coli* spp.	SAL1+E
	SAL 3+E

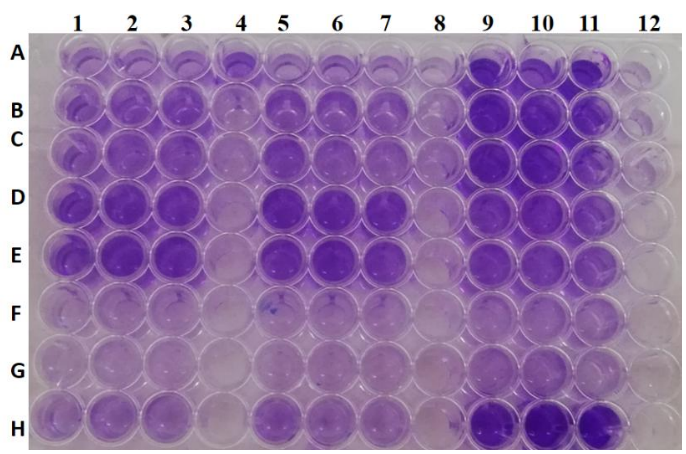

Fig. 1. Arrangement of biofilms formed by different bacterial cultures in Microtitre plate following the staining procedure. (Column 4, 8 and 12 are the negative controls and other wells indicate the presence of biofilms)

2.4 Statistical Analysis

Biofilm-forming ability was compared by analyzing the degree of biofilm formation differences using two-sample t-test, one-way ANOVA and Duncan's multiple range tests in SAS software version 9 (SAS Institute, Inc., Cary, NC, USA).

3. Results and Discussion

This study investigated the biofilm formation ability of *Salmonella*, *Proteus* and *E. coli* when they are present as mono species. Further, it investigated the biofilm formation ability when the *Salmonella* interacts with other spp such as *Proteus* and *E. coli* in the form of dual biofilm.

3.1 Mono Biofilm-Forming Ability of *Salmonella*, *Proteus* and *E. coli*

Salmonella, *E. coli* and *Proteus* species as mono biofilms showed different biofilm-forming abilities throughout the tested time. At 24 hours, both *Salmonella* 1 (SAL 1) and *Proteus* formed more biofilms than those formed by *E. coli* and *Salmonella* 3 (SAL 3) ($P \leq 0.05$). However, SAL 3 showed the lowest biofilm-forming ability at 24hr (0.876±0.065), and it was not significantly different from biofilm formed by E. *coli* (1.002±0.034) ($P \geq 0.05$). Similarly, at 48hours, SAL 1 (2.332±0.2) and *Proteus* spp. (2.513±0.227) showed significantly higher biofilm-forming abilities than that of SAL 3 (1.506±0.287) and *E. coli* (1.148±0.279) (Table 2).

At 72hr SAL 1and *Proteus* formed higher biofilm than that of the *E. coli* and Sal 3 ($P \leq 0.05$) (Table 2). The observed optical density values of SAL 1 was 2.593±0.184 and *Proteus*had 1.969 ±0.048, followed by *E. coli* 1.198 ± 0.640 and *Salmonella* 3 (SAL 3)0.891±0.052. Though the SAL 3 was the lowest biofilm former at 72hr, that was not significantly different from *E. coli* ($P \geq 0.05$).

At 96hr similar to the 72hrs, the significantly higher biofilm formation was investigated in both SAL 1 (2.022±0.216) and *Proteus* (2.195 ± 0.068), which was higher than the SAL 3 (1.110 ± 0.115) and *E. coli* (1.388 ± 0.248) ($P \leq 0.05$). Although SAL 3 showed the lowest biofilm-forming ability at 96hr, that wasn't significantly different from the biofilm-forming ability of *E. coli* ($P \geq 0.05$). At 120 hours SAL 1, *Proteus* and *E. coli* had significant higher biofilm-forming abilities (2.203±0.283, 2.123±0.219 and 1.821±0.166 for SAL 1, *Proteus* and *E. coli* respectively) than that showed by SAL 3 (2.123±0.219) ($P \leq 0.05$) (Table 2).

A study was done by Kwiecinska-Piróg [21] and the group in 2014 showed that *Proteus* spp. are forming strong biofilms as detected by 2,3,5-triphenyl-tetrazolium chloride-based assay, and this finding is in line with the current study, which showed higher biofilm formation by *Proteus* spp. Isolated from broiler chicken meat. Supporting the current study, Wilks et al. [22] revealed the increasing cell at 24hr with pseudo three-dimensional structures [22]. Similarly, the higher biofilm-forming ability of *Proteus* mono biofilm at 24hr on LB broth was also investigated with mushroom type architecture by Jones et al. [23]. According to Fernández et al. [24], clinical *Proteus* strains showed denser biofilm with more extracellular polymeric substance production. Also, it sowed higher fimbriae production ability which may cause the higher initial attachment of *Proteus* biofilms at 24hr [24]. The higher biofilm-forming ability of Proteus may be affected by its higher capability of extracellular matrix production, nutrient channel formation, and fimbriae production ability. Throughout the entire tested time durations, SAL 1 showed higher biofilm formation as aforementioned. The difference in the biofilm-forming ability of SAL 1 and SAL 3 as mono biofilms may be due to their serovars variations. However, the significantly different biofilm-forming abilities among serovars were also investigated by Vestby et al. [25] and Chelvam et al. [26]. Among tested *Salmonella* serovars, Chelvamet al. [26] investigated swarming motility variation, i.e., some serovars with swarming motility. While some were not, that affected virulence and early stages of biofilm formation [27]. In the case of *E.coli* biofilms, different pathotypes have numerous biofilm-forming abilities. In this sense, some pathotypes t with gene expression related to biofilm formation such as agn43 and fimH, absence of curli and fimbriae, and absence of motility behavior cause weak biofilm-forming ability [28].

When considering the biofilm-forming ability during the period of 120 hours, the biofilm-forming ability of *Proteus* spp. and *Salmonella* isolate 3 (SAL3) has reached its maximum at 48 hours. The optical density of the biofilm cells were 2.513±0.227 and 1.506±0.287 for *Proteus* spp. and SAL 3, respectively. After 48 hours, the biofilm formed by *Proteus* and SAL 3 started to decline, and at 72 hours, it reached its minimum biofilm cells with the absorbance values of

Table 2. Biofilm-forming ability of *Salmonella*, *Proteus* and *E. coli* as mono species

Isolates	Optical density at different time points				
	24 hr	48 hr	72 hr	96 hr	120 hr
***Salmonella* (SAL 1)**	***1.546±0.306[a]***	***2.332±0.200[a]***	***2.593 ±0.184[a]***	***2.022±0.216[a]***	***2.203±0.283[a]***
***Salmonella* (SAL 3)**	0.876±0.065[b]	1.506±0.287[b]	0.891±0.052[b]	1.110 ±0.115[b]	1.255±0.042[b]
***Proteus* spp.**	***1.647±0.298[a]***	***2.513±0.227[a]***	***1.969 ±0.048[a]***	***2.195 ± 0.068[a]***	***2.123±0.219[a]***
E. coli	1.002±0.034[b]	1.148±0.279[b]	1.198±0.640[b]	1.388 ±0.248[b]	***1.821±0.166[a]***

**Data were presented as the mean ± standard deviation. Means with different superscripts in the same column are the significant difference (Bold and italic showed the highest biofilm formation at each time point)*

Proteus 1.969±0.048, SAL 3 0.891 ± 0.052 respectively (Fig. 2). Interestingly by 96hours, SAL3 again started to increase the biofilm cells (2.022±0.216) and continued to increase until the end of the experimental period, i.e.,120hours (2.203±0.283) (Fig. 2). Another *Salmonella* isolate (SAL1) exhibited the highest biofilm formation at 72hours (2.593 ±0.184); afterward, it declined to have the lowest biofilm at 96hours (2.022±0.216) and regained its increasing biofilm-forming ability at the end of 120hr (2.203±0.283). Contrary to the other isolates, *E. coli* showed a gradual increase in the biofilm cells from the beginning of the experiment. Ia gradual increase in the biofilm cells from the beginning of the experiment. It continued to increase until the end of the experimental period (Fig. 2).

However, previous findings also revealed different biofilm-forming abilities in different tested pathotypes [27,29]. The current study findings of SAL 1 and SAL 3 showing different biofilm-forming abilities may vary their pathotypes. Current study findings of the optimum biofilm-forming ability of *Salmonella* isolate (*Salmonella* 3) at 48hr also agreed with several previous findings [30,31,32]. Among those findings, Shatila et al. [32] has observed more prominent curli and cellulose production at 48hr. Curli and cellulose overexpression accounts for thicker biofilm formation in *Salmonella* species [33]. Hence the maximized biofilm-forming ability at 48hr may be due to their higher expression of curli and cellulose production ability.The declining biofilm-forming ability of some *Salmonella* pathotypes at 72hr described by Agarwal et al., [30].This may result from a nutrient depletion in extended incubation time durations, leading to biofilms' dispersal [34]. A previous research study which was done by Rodríguez-Melcón et al. [35] agreed with the findings of the current study having the highest biofilm-forming ability of SAL 1 at 72hr. Rodríguez-Melcón and his team also have investigated the increasing biofilm-forming trend of Salmonella species from 48hr to 72hr. Apart from that, the progression of biofilm formation since 2 to 4 days is affected by their increasing pellicle forming ability with extending incubation time [25], which supports current findings of increasing biofilm formation of SAL 1 isolate at 72hr.The minimum biofilm-forming ability of SAL 1, SAL 3 and *Proteus* could be affected by entering bacterial biofilm cells into viable but non-culturable stage [36,37] followed by repeat increment at extended post-incubation could also be happened their stress adaptation technique [38]. These VBNC can be investigated using standard plating techniques [39], not by microtiter plate assay. Thus lower absorbance could be recorded in a method such as microtiter plate assay as the current study investigated.

In case of biofilm-forming ability of *Proteus* at 48hr with increasing extracellular matrix component *also* speculated by [40]. However, the speculated continual increment of *Proteus* biofilm development even at 7 days of post-incubation. Moreover, at 96hr, *Proteus* tend to form more organized biofilm architecture [24], which supports the current study findings of higher biofilm-forming ability than that showed at 72hr. Further that enhancing the biofilm-forming ability of *E. coli* as the mono biofilm is also dependent on temperature, whereas, under low incubation, temperature poses to enhance the biofilm-forming ability of *E. coli* species [41]. Moreover, some prior findings have similar results as the continuous increasing trend of the biofilm-forming ability of *E. coli* by several research groups [42,43,44,45]. The enhanced motility behavior of *E. coli* under extended time points also increases the initial attachment and biofilm formation process [42]. Apart from the incubation time, *E. coli* biofilm formation is regulated by several intrinsic factors such as strain diversity, nutrient availability, cellular structures curli/fimbriae, and gene expression patterns [45,46,47].

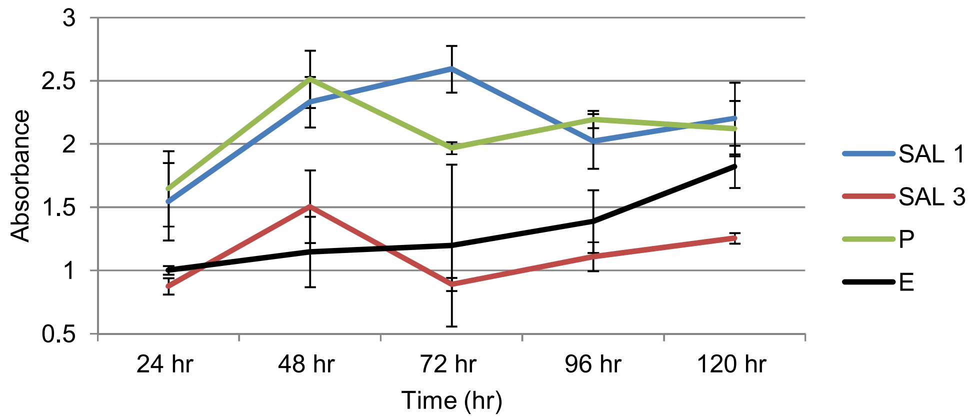

Fig. 2. Biofilm-forming ability of single isolates as mono-biofilm during the period of 120hrs
**SAL1- Salmonella 1, SAL 3- Salmonella 3, P- Proteus, E- E. coli*

3.2 Interaction of *Salmonella* 1 (SAL1) with *Salmonella* 3 (SAL 3), *Proteus* and *E. coli* in Dual Biofilm Formation

At 24 hours, *Salmonella* 1 (SAL1) and combinations of *Salmonella*(SAL1) with SAL 3, *Proteus* and *E. coli* have not shown any significant differences in biofilm-forming ability($P \geq 0.05$) (Table 3). *Salmonella* isolates1 (SAL1) alone had an OD value of 1.590±0.111, and its interactions SAL 1+SAL 3, SAL1+P &SAL 1+E showed OD values of 1.549±0.104, 1.546±0.306 and 1.505±0.090, respectively (Table 3). At 48hr SAL 1+SAL 3 combinations showed significant lower biofilm-forming ability with 1.901±0.187 absorbance value than that of SAL 1 (2.332±0.200) and its other interactions, SAL 1+P (2.508±0.005) SAL 1+E (2.343±0.006) respectively (Table 3). Similarly, at 72hr, SAL 1 interaction with SAL 3 (SAL 1+SAL 3) formed significantly less biofilms (2.101±0.145) than that of SAL 1 as mono biofilm (2.593±0.184) and SAL 1 as dual biofilms with *Proteus* (SAL 1+P;2.375±0.047) and *E. coli* (SAL 1+E;2.448±0.148). Contrary to that, SAL 1 and its interactions of SAL 3 (SAL 1+SAL 3), *E. coli* (SAL 1+ E) and *Proteus* (SAL 1+P) showed similar biofilm-forming abilities at 96hr, without causing any significant differences ($P \geq 0.05$). That biofilm-forming abilities were (SAL 1) 2.022±0.216, (SAL 1+SAL 3) 1.862 ±0.154, (SAL 1+P) 1.916 ±0.165 and (Sal 1+E) 1.825 ± 0.172 correspondingly (Table 3). At 120 hours, similar to the 96-hour time point, SAL 1 and its interactions did not show any significant increment or reduction in biofilm formation. At 120hr shown by the SAL 1 and its interactions was 2.203 ±0.283for SAL 1, 2.148 ±0.127 for SAL 1+SAL 3,2.293±0.071 for SAL 1+P and2.225 ± 0.09 for SAL 1+E (Table 3).

The significantly lower biofilm-forming ability of SAL 1 interaction with SAL 3 at some tested time points (48hr, 72hr), maybe due to the lower biofilm-forming ability of SAL 3, which showed at its monoculture status (Fig. 1). The suppressive action *Salmonella* in dual biofilm formation was also described by Esteves et al. [48] and described the poor outcompete manner of *E.coli* over the *Salmonella* strains. The significantly less biofilm-forming ability of SAL 1+SAL 3 as dual culture also corroborates with previous findings of Gkana et al. [49] and Frozi et al. [50], who speculated the lower biofilm-forming ability of *Salmonella* as dual cultures. However, the observed low biofilm capabilities or same biofilm capabilities of *Salmonella* and its interaction in different time points (Table 3) may be due to strain-dependent different properties, such as EPS production, presence of either flagella or fimbriae, etc. [51].*Salmonella* strains, *S.* Heidelberg, *S.*Hadar, and *S.* Typhimurium, were weak biofilm producers on microtiter plates. The cellular appendages curli and fimbriae positive strains also increase the attachment process than negative strains [52]. Apart from that, *Salmonella* strains and *E. coli* strains which are negative curli, fimbriae, and cellulose producers, have also been investigated with less cell percentage than the curli and fimbriae positive stains [53]. So with those investigations, current study findings of low/same biofilm-forming capabilities may be due to the absence of cellular structures in *Salmonella* 1, such as curli and fimbriae in tested strains.

Table 3. Interaction of *Salmonella* isolate 1 (SAL 1) with *Proteus* and *E. coli* in biofilm formation as dual species

Isolate / combinations	Optical density at different time points				
	24 hr	48 hr	72 hr	96 hr	120 hr
SAL 1	1.590±0.111[a]	2.332±0.200[a]	2.593±0.184[a]	2.022±0.216[a]	2.203 ±0.283[a]
SAL 1+ SAL3	*1.549±0.104[a]*	***1.901±0.187[b]***	***2.101±0.145[b]***	*1.862±0.154[a]*	2.148 ±0.127[a]
SAL1+ P	1.546±0.306[a]	2.508±0.005[a]	2.375±0.047[a]	1.916±0.165[a]	2.293±0.071[a]
SAL 1+ E	1.505±0.090[a]	2.343±0.006[a]	2.448±0.148[a]	1.825±0.172[a]	2.225 ± 0.09[a]

**Data were presented as the mean ± standard deviation. Means with different superscripts in the same column are a significant difference. (SAL 1- Salmonella 1, SAL 1+SAL 3- Salmonella 1 interaction with Salmonella 3 as dual biofilm, SAL 1+P- Salmonella 1 interaction with Proteusas dual biofilm, SAL 1+E- Salmonella 1 interaction with E. coli as dual biofilm), Bold and italic showed the lowest biofilm formation at each time point*

SAL 1 and its all interactions showed the lowest biofilm-forming abilities at 24hr than that showed at other time durations. In case of SAL1 together with *Proteus* spp. (SAL1 +P) showed the highest biofilm-forming ability at 48hr, with its highest optical density value of 2.508±0.005 and then declined at 72hr (2.375±0.047), 96hr (1.916 ±0.165), which followed regains its biofilm-forming ability at 120hr (2.293±0.071). The biofilm-forming trend of the other two interactions (SAL 1+SAL 3, SAL 1+E) and SAL1 mono biofilm showed similar biofilm-forming trends throughout the tested time durations. In context, SAL 1 mono biofilm increased its biofilm-forming ability at 48hr (2.332±0.200) and 72hr (2.593±0.184), which declined at 96hr (2.022±0.216), followed by increment at 120hr (2.203 ±0.283). The highest biofilm formation of that SAL 1 mono biofilm showed at 72hr among absorbance mentioned above values of tested different time points (Fig. 3). Relatively to that, SAL 1+SAL 3 also enhanced its biofilm-forming ability from 24hr (1.549±0.104) to 72hr with its highest absorbance (2.101±0.145), declined at 96hr (1.862 ±0.154) and enhanced again at 120hr (2.148 ±0.127). Among that absorbance values, SAL 1+SAL 3 attained its highest biofilm formation at 120hr (Fig. 3). In the case of SAL 1 interaction with *E. coli* as dual biofilm, it had increased its biofilm-forming ability from 24hr (1.505±0.090) to 72hr (2.448±0.148), followed by declining at 96hr (1.825 ± 0.172) and repeatedly increased its biofilm-forming ability at 120hr (2.225 ± 0.09). Anyhow, among those values, SAL 1+E has attained its highest biofilm formation at 72hr (Fig. 3).

The progressive development of *Salmonella* biofilm i.e., SAL 1, SAL 1+E at 72hr, also agreed with previous findings, which elucidated that the greatest thickness has been investigated and followed by decreasing biovolume at extended incubation. However, biofilm-forming abilities may be due to the decreasing matrix component at extending time durations [54]. But in SAL 1+P combination, highest biofilm formation at 48hr, as a different observation than other interactions' maximal points, which may be due to increased extracellular matrix production of *Proteus* in some extended time durations [40]. The lower absorbance value at 96hr could be affected by entering bacterial biofilm cells into a viable but non-culturable state under nutrient-depleted conditions [36,37]. Collectively this repeated increment of dual biofilms may be due to the rapid growth of *Salmonella*, *E. coli* biofilm in extended time points and more surface coverage with irregular complex biofilm structure and higher exopolymer production [51]. As that cells can be detected using standard plating techniques [39], the low absorbance values could be recorded at 96hr in microtiter plate readings, followed by stress adaptation [38]. The repeat increment of the biofilm-forming ability of all tested combinations at 120hr could have appeared as the long-term survival of *Salmonella* species with stress adaptation and predominant radars morphotype [29]. The radar morphotype has appeared due to biogenesis curli and cellulose, which are important in the biofilm formation [55]. Hence this long-term survival may also be affected by curli and fimbriae production too.

3.3 Interaction of *Salmonella* 3 (SAL3) with *Salmonella* 1 (SAL 1), *Proteus* and *E. coli* in Dual Biofilm Formation

The experiment conducted to see the interaction of SAL 3 with other organisms (SAL 3, *Proteus,* and *E. coli*) showed that at 24 hours, SAL 3 interaction with SAL 1 as dual biofilm (SAL 1+ SAL3) had significant higher biofilm-forming ability (1.549±0.104) than SAL 3 alone in the mono biofilm (0.876±0.065). Apart from that, SAL

3 interaction with *Proteus* spp. (SAL 3+P) also showed significantly higher biofilm formation (1.126±0.173) than the SAL 3 existent as mono biofilm (0.876±0.065), but that was (SAL 3+P) significantly lower than the SAL 1+SAL 3 interaction (1.549±0.104). However, SAL 3 as mono biofilm (0.876±0.065) and interaction with *E. coli*, as dual biofilm (SAL 3+E) had similar biofilm (1.038±0.07) forming abilities, without significant differences at 24hr ($P \geq 0.05$). At 48hr, SAL 1+SAL 3 interaction showed significantly higher biofilm-forming ability (1.901±0.187) than that shown by SAL 3 (1.506±0.287) alone. Apart from that, the biofilm formed by SAL 3 alone and interaction with *E. coli* (SAL 3+E) has not shown any significant difference in biofilm formation at 48hr. At 72hr, the SAL 1 interaction with SAL 3 as dual biofilm formed a higher biofilm (2.101±0.145) than SAL 3 alone, and with all other combinations (SAL 3+P, SAL 3+E). At 96hr also SAL 1+SAL 3 showed more biofilms (1.862 ±0.154) than SAL 3 (1.110 ± 0.115) monoculture counterparts ($P \leq 0.05$) (Table 4). At 96hr, biofilm formation by SAL 3 together with *Proteus* spp. (SAL 3+P)showed significantly lower (1.594 ±0.160) biofilm than SAL 1 interaction with SAL 3 (1.862 ±0.154), whereas the SAL 3 alone had the lowest biofilm at 96 hours. At 96hr, biofilm formed by SAL 3 and *E. coli* was not significantly different from the biofilm formed by SAL 3 alone (Table 4). At 120hr, SAL 1+ SAL 3 as dual biofilm former achieved its significantly higher biofilm formation (2.148 ±0.127) compared to SAL 3+P dual interaction had the similar biofilm-forming ability as showed by SAL 3 alone without any significant difference ($P \geq 0.05$). However, SAL 3+E formed significantly fewer biofilms than theSAL 3 mono biofilm counterpart ($P \leq 0.05$). SAL 3 isolate significantly increased its biofilm-forming ability at all tested time points after co-culturing with SAL 1 (SAL 3+SAL 1) than that shown by SAL 3 mono culture counterpart (Table 4). The highest biofilm-forming ability of SAL 3+P over SAL 3, is in agreement with previous findings [35,56,57]. Among them, Rodríguez has described the increasing *Salmonella* biofilm-forming ability with the presence of other bacterial species. This may be due to the spatial different distribution patterns of species within biofilm architecture. Moreover, the outcompete behavior of Proteus in dual biofilms is also described by previous findings [56,57]; hence, this higher biofilm formation could be due to the latter to *Proteus* outcompete behavior in dual biofilm too. Higher biomass of *Proteus* dual culture biofilms also resultant as enhancing EPS production ability [58]. As another factor, the strain differences in the biofilm-forming ability of isolates [59] could be a major cause for the deviation of significantly higher biofilm formation in some point tested time points. In the case of SAL 3+E, lower biofilm-forming ability than the SAL 3 mono biofilm at 120hr may be due to *E. coli* metabolite indole, which acts as a

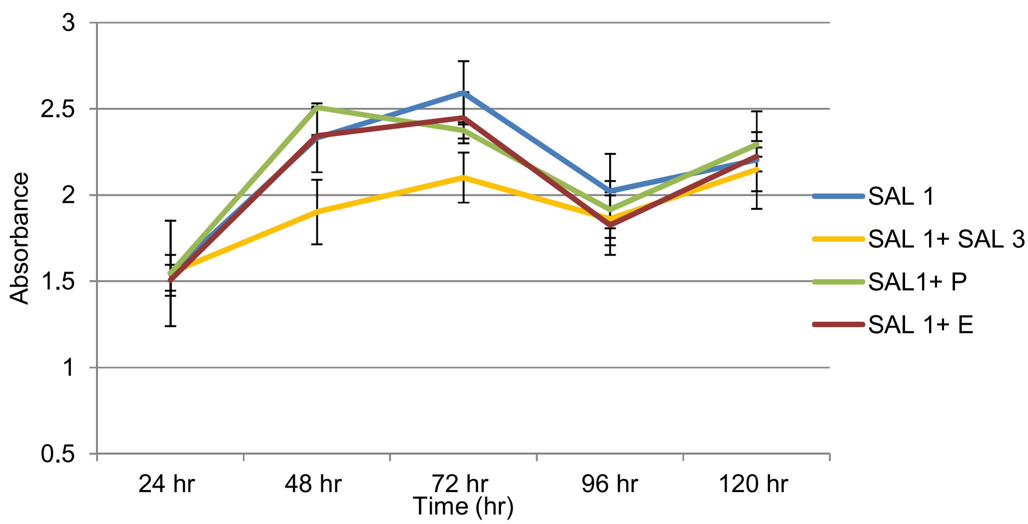

Fig. 3. Biofilm formation of *Salmonella* isolate 1 (SAL 1) and its combination with *Proteus* and *E. coli* as dual species during the period of 120hrs

**SAL 1: Salmonella 1, SAL 1+SAL 3: Salmonella 1 interaction with Salmonella 3 as dual biofilm, SAL 1+P-Salmonella 1 interaction with Proteus as dual biofilm, SAL 1+E- Salmonella 1 interaction with E. coli as dual biofilm* suppressive factor of biofilm formation. This lower biofilm-forming ability may be affected by indole metabolite produced by *E. coli* strains which negatively correlates with the biofilm formation process [60]. Under the presence of indole in *E.coli* biofilms, architectural deviations of tower colonies to flat colonies have been exhibited by Lee et al. [61]. However, this suppressive effect was absent in earlier tested time points. In this sense, *E. coli* rapid biofilm-forming ability, which was highest at 120hr than other tested time duration, could be a reason for increasing toxic metabolite indole, leading to the prominent suppressive effect of dual interactions. Apart from that, valine, a metabolite by *E. coli*, is also impaired on inhibitory activities of other bacterial strains [62].

The higher biofilm-forming ability of SAL 3 interaction with SAL 1 than SAL 3 mono biofilms at all tested may be due to comparative higher biofilm-forming ability of *Salmonella* 1 as mono biofilm, which accelerates the lower biofilm-forming ability of *Salmonella* 3.EPS production abilities of *Salmonella* strains greatly affected their biofilm-forming abilities. In contrast, EPS positive strains produce more biofilms than negative strains. Apart from that, EPS negative strains poor biofilm-forming ability is also stimulated by EPS positive strains. Hence the current finding of higher biofilm formation of *Salmonell*a co-cultures could be their different EPS production ability [27].

Except for the SAL 3 interaction with SAL 1 (SAL 1+SAL 3), all other interaction with SAL 3 has shown a similar trend in biofilm formation (Fig. 4) throughout the time period. SAL 1+SAL 3 interactions have gradually shown an increment of biofilm from 24hours (1.549±0.104), and it reached its maximum at 72hr (2.101±0.145). It was declined at 96hr to its minimum value (1.862 ±0.154), and there was a second wave of increment afterward increasing at 120hr (2.148 ±0.127) (Fig. 4).

When considering *Salmonella* isolate 3 (SAL 3), it also showed the trend of gradual increment of biofilm formed from 24hr (0.876±0.065), and it reached its maximum level at 48hr, with the highest absorbance (1.506±0.287) followed by declining to its lowest value at 72hr (0.891±0.052). Afterward, this has shown the second wave of an increment in the biofilm at 96 hr (1.110 ± 0.115) and 120hr (1.255± 0.042), respectively (Fig. 4).

The biofilm formation trend of SAL 3+P combination also increased from 24hr (1.126±0.173) to 48hr, where the highest absorbance value (1.621±0.095) was found. Afterward, it was declined to reach it minimum at 72hr (1.185±0.243) followed by increment at 96hr (1.594 ±0.160) and reduced at 120hr to its minimum value (0.9649±0.378). Interestingly, SAL 3 interaction with *Proteus* (SAL 3+P) exhibited two prominent peaks at 48hr and 96hr, respectively (Fig. 4).

Interaction of SAL 3 with *E. coli* (SAL 3+E) also showed a similar pattern with others having enhancement of biofilm-forming ability from 24hr (1.038±0.070) to 48hr with its highest biofilm formation at (1.337±0.039). The biofilm formed was declined at 72hr, reaching its lowest I value of 1.015±0.221. Different from all the other combinations tested in this study, this interaction of SAL3 and *E. coli* has shown a continuously increasing trend of biofilm formation after 72hours (from where the minimum value), having biofilms of 1.159 ± 0.135 at 96hr and 1.164 ± 0.110 at120hr (Fig. 4).

Table 4. Interaction of *Salmonella* isolate 3 (SAL 3) with *Proteus* and *E. coli* in the formation of biofilm as dual species

Isolate / combinations	Optical density at different time points				
	24 hr	48 hr	72 hr	96 hr	120 hr
SAL 3	0.876±0.065^{c}	1.506±0.287^{b}	0.891±0.052^{b}	1.110±0.115^{c}	1.255± 0.042^{b}
SAL 1+ SAL 3	***1.549±0.104^{a}***	***1.901±0.187^{a}***	***2.101±0.145^{a}***	***1.862 ±0.154^{a}***	***2.148 ±0.127^{a}***
SAL 3+ P	1.126±0.173^{b}	***1.621±0.095^{ab}***	1.185±0.243^{b}	1.594 ±0.160^{b}	0.9649±0.378^{b}
SAL 3+ E	1.038±0.070^{bc}	1.337±0.039^{b}	1.015±0.221^{b}	1.159±0.135^{c}	1.164±0.110^{c}

**Data were presented as the mean ± standard deviation. Means with different superscripts in the same column are significantly different*

**SAL 3- Salmonella 3 mono biofilm, SAL 1+SAL 3-Salmonella interaction with Salmonella 3 as dual biofilm, SAL 3+P- Salmonella 3 interaction with Proteusas dual biofilm, SAL 3+E- Salmonella 3 interaction with E. colias dual biofilm.Bold and italic showed the highest biofilm formation at each time point*

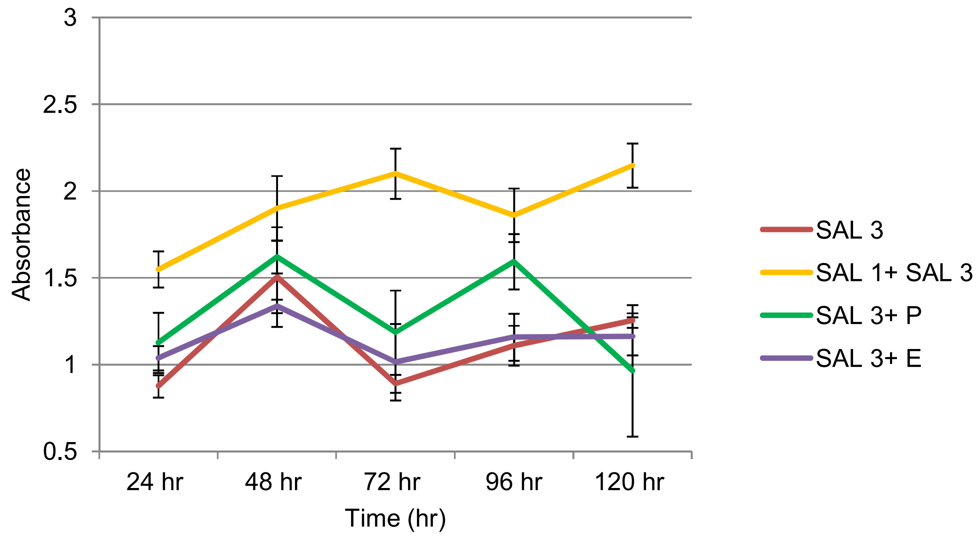

Fig. 4. Biofilm formation of *Salmonella* isolates 2 (SAL 3) and its combination with *Proteus* and *E. coli* as dual species during the period of 120 hrs

******SAL 3*- *Salmonella 3 mono biofilm, SAL 1+SAL 3- Salmonella 1 interaction with Salmonella 3 as dual biofilm, SAL 3+P- Salmonella 3 interaction with Proteus as dual biofilm, SAL 3+E- Salmonella 3 interaction with E. coli as dual biofilm*

Higher biofilm-forming ability of *Salmonella* at 48hr was also previously investigated by Sexias et al. [63] and suggested that it may have appeared with a gradual increment of viable cell count. That study further supports our current findings of the declining biofilm-forming ability of tested interactions at 72hr, and the reason behind that could be an increase in the production of toxic metabolites. Most studies investigated higher biofilm formation in nutrient nutrient-depleted conditions [64,65], so in the current study at 96hr and 120hr repeated increment of *Salmonella* 3 and *E. coli* dual biofilm may be due to the adaptation for limited nutrient depletion. The biofilm formation is affected by different EPS-producing patterns shown by *Salmonella* and *E. coli* species.

In contrast, *Salmonella* species achieve the highest biofilm formation with the presence of curli and cellulose. The highest percentage of curli-producing bacteria has also been recovered from mixed biofilms [51]. Hence these different biofilm-forming abilities of *Salmonella* and *E. coli* dual biofilms, which was higher at 48hr, may be due to variation of extracellular matrix component production. In line with that higher biofilm-forming ability of *Salmonella* as dual biofilm in extended time durations than that showed by its mono biofilm also previously investigated by Iñiguez-Moreno et al. [66] and further investigated prominent growth may be affected by increasing matrix carbohydrate and protein fractions in *Salmonella* dual biofilms than its monoculture counterparts.

4. CONCLUSION

This study concluded that there is a variation among isolates and their combinations in forming the biofilms, where there is an enhancement of biofilm in dual-species over the mono-species in some interaction, and there is a reduction in biofilm formation by dual-species with some combinations. Further, this concluded that there is an interaction of *Salmonella* with other commonly found bacteria such as *Proteus* and *E. coli* in biofilm formation.

DISCLAIMER

The products used for this research are commonly and predominantly used in our research area and country. There is absolutely no conflict of interest between the authors and producers of the products because we do not intend to use these products as an avenue for any litigation but for the advancement of knowledge. Also, the research was not funded by the producing company rather, it was funded by the personal efforts of the authors.

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

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