International Journal of Pathogen Research

Volume 13, Issue 6, Page 145-159, 2024; Article no.IJPR.127940
ISSN: 2582-3876

A Novel Study of Shigella Biosurfactants Like Molecule Produced and Assessed Anaerobically

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Authors' contributions

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

Article Information

DOI: https://doi.org/10.9734/ijpr/2024/v13i6329

Open Peer Review History:
This journal follows the Advanced Open Peer Review policy. Identity of the Reviewers, Editor(s) and additional Reviewers, peer review comments, different versions of the manuscript, comments of the editors, etc are available here: https://www.sdiarticle5.com/review-history/127940

Original Research Article

Received: 07/10/2024
Accepted: 10/12/2024
Published: 12/12/2024

ABSTRACT

Shigella has settled on extremely sophisticated mechanisms to disrupt host cell progressions to endorse infection, escape immune exposure, and avoid bacterial clearance. The design of new

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Cite as: *Kayath, Christian Aimé, Nicole Prisca Makaya Dangui Nieko, Duchel Jeanedvi Kinouani Kinavouidi, Christ Dieuveil Bayakissa Malanda, and Etienne Nguimbi. 2024. "A Novel Study of Shigella Biosurfactants Like Molecule Produced and Assessed Anaerobically". International Journal of Pathogen Research 13 (6):145-59. https://doi.org/10.9734/ijpr/2024/v13i6329.*

therapeutic targets for the optimal management of Shigella infection must give a follow-up action target in the live anaerobic cycle. The aim of this present work is to explore the ability of *Shigella* to produce and secrete biosurfactant like molecules anaerobically. This study describes the isolation of 93 *Shigella* spp. candidates, from hospital and environmental samples. These isolates, plus lab strains, were tested in a comprehensive in vitro screening regime including biochemical and microbiological analysis, emulsification index (EI24), the DNA technology and sequencing, swarming test, the biofilm formation and *S. flexneri* were able to secrete and produce biosurfactant with the percentage ranging from 68 to 100%. Strains were also swarming in semisolid media, with swarm diameters ranging from 75 to 85 mm on soft agar (0.5%) in the absence of oxygen. This study showed that *Shigella* were unable to invade eggs in the absence of oxygen, with zero (0%) invasion rates, but retained their ability to form biofilms. The invasion rate was zero in anaerobiosis and more than 50% in aerobiosis. These results show that biosurfactants are produced in cotranslational pathways and are secreted into the extracellular medium through the *Shigella* type three secretion system (T3SS). *Shigella* biosurfactants are involved in biofilm formation and secrete into the extracellular medium via the phenomenon known as the "T3SS leakage."

Keywords: Shigella; pathogenicity; anaerobiosis; biosurfactant; T3SS.

1. INTRODUCTION

The pathogenicity of enterovirulent bacteria is due to the fact that they colonise various sites in the human gut. Enteropathogenic *E. coli* (EPEC) [1,2], Enterotoxigenic *E. coli* (ETEC) and diffusely adherent *E. coli* (DAEC) preferentially attack the small intestine, while *Shigella* spp., *Campylobacter* spp., Enterohaemorrhagic *E. coli* (EHEC) [3], and Enteroinvasive *E. coli* (EIEC) attack the large intestine [4].

Shigella spp. are strictly pathogenic, facultatively aero-anaerobic, immobile, nonencapsulated, spore-free bacteria that measure 2 to 3 µm long by 0.5 to 0.7 µm wide and are members of the Enterobacteriaceae family. *Shigella* spp. do not ferment lactose or ferment slowly [5]. They are responsible for shigellosis, which is an intestinal infection that mostly affects the large gut, where the bacteria multiply and cause inflammation of the mucous membranes, as well as bloody, milky diarrhoea. Tropical countries, including the Republic of Congo, continue to experience this infection, especially during the warmer and colder seasons [6,7].

In addition to the biosurfactant and lipopolysaccharides on the outer membrane of *Shigella* spp., other components involved in pathogenicity include adhesins, invasins, toxins, protein secretion system, and iron absorption system [7]. *Therefore, Shigella* spp. are enteroinvasive bacteria, capable of penetrating the epithelial cells of the intestinal mucosa and multiplying there, leading to the formation of abscesses and ulcerations [8]. These bacteria do not enter the general circulation, but local phenomena can be accompanied by toxicosis and dehydration [9]. The invasive phenomenon affects the superficial mucosa and decreases to the deeper layers [10]. These germs can cause widespread epidemics or sporadic cases and are only found in humans [7].

In terms of global epidemiology, *Shigella* is the second most common cause of death from rotavirus-related diarrhoeal disease in children under the age of five years of age. In reality, it results in nearly 164300 deaths annually throughout the world, 54900 of which occur in sub-Saharan Africa and South Asia [11]. There are no epidemiological data on this disease available for the Republic of Congo.

Shigella is capable of entering under anaerobic conditions and forming biofilms, which in some circumstances allows it to cross the digestive tract and reach the colon where the infection occurs [12]. This pathogenicity profile is defined by its type 3 secretion apparatus, which allows the delivery of virulence effectors to the host epithelial cell.

According to the most recent findings of ongoing clinical trials, there has not been much progress in developing a vaccine that can effectively prevent *Shigella* infection. On the other hand, *Shigella* spp. strains that are resistant to a number of antibiotic families, including -lactams, quinolones, and aminoglycosides, are becoming increasingly common... [13].

It has recently been shown that all *Shigella* spp. strains isolated from the environment, including *Shigella flexneri*, *Shigella boydii*, *Shigella sonnei*,

and others, have the ability to produce and secrete biosurfactants into the extracellular medium. The same study demonstrated that biosurfactant was secreted through the type 3 secretion system under leaky and induced conditions. It was postulated that Shigella could use biosurfactant to invade the epithelial cell [14]. The Type Three secretion system has also been demonstrated to be oxygen dependent [15]. It does not function in anaerobic environments. In these circumstances, *Shigella* cannot invade and deliver virulence effectors to the host epithelial cell [15]. However, the ability to produce biosurfactants in preinvasion and proinvasion situations has only been tested under aerobic conditions [14]. This work aims to explore the ability of Shigella spp. To secrete a biosurfactant like a molecule aerobically. This research could contribute to understanding of the invasion mechanism of epithelial cells by bacteria of the genus *Shigella*.

2. METHODOLOGY

2.1 Sampling, Strains, and Culture Conditions

To sample wastewater, fecal-contaminated soil, and water sources, four Brazzaville (Republic of Congo) districts have been targeted, including Makélékélé, Bacongo, Poto-Poto, and Madibou. Three samples per sampling sites. The samples were packaged in a plastic bag and then transported to the laboratory. Once in the laboratory, the samples were stored at +4°C for immediate experimentation. Successive dilutions were carried out. EMB, SS, and Hektoen media have been used for *Shigella* spp. For environmental strains, five (5) main collection sites and for clinical strains, one site, isolates were collected. In total, we collected three (3) clinical strains, 43 (43) isolates from faecal-contaminated soils, and 47 (47) isolates from wastewater. Four (4) laboratory strains (S. *flexneri* [16], *S. flexneri* Spa40- (kanamycin resistant) [17], *S. sonnei* [18], and *S. boydii*) were used as reference strains. The *S. flexneri* M90T-Sm (Sm^r) has been previously described [16]. The Petri dishes were incubated at 37°C for 24 h. After the first isolation in Petri dishes, different colonies were obtained. Each characteristic colony from SS was isolated separately isolated and plated with LB with streptomycin 100µl/ml or kanamycin 50µg/ml. The purification of the isolates was rigorously carried out by successive and alternating subcultures on Hektoen Enteric Agar (HE). The purity was estimated by using a microscope for morphological characterisation. Gram status was determined by using 3% KOH.

2.2 Identification of Isolates and Genomic DNA Extraction and RFLP-PCR

To confirm the *Shigella* isolate, genomic DNA extraction and purification was performed using the NucleoSpin Microbial DNA kit (Macherey-NAGEL). Briefly, the targeted isolate was grown in 5 ml of LB broth for 24 h at 37°C with stirring. DNA purity was assessed by electrophoresis on 1% agarose gel and by the optical density ratio of 260/280 nm. The 16S rRNA gene has been amplified by PCR (Thermal Cycler, Bio-Rad) using universal primers fD1 (5 ′-AGACTTTGATCCTGGCTCAG-3 ′) and rP2 (5′-ACGGCTACCTTGTTACGACTT-3′). 5 µL of each amplification product was mixed with 2 µL of loading buffer (BIOKÉ). The mixtures were subjected to electrophoresis on 1% agarose gel (w/v). The 10 kb 2-Log (BIOKÉ) was used as a molecular weight marker. The PCR products were purified using the gel extraction kit solution (Omega Bio-tek), and digested using restriction enzymes (EcoRI and PstI). The digestion profiles were compared with those of reference strains of *Shigella* that had already been identified. This showed that the isolated strains are members of a particular bacterial genus [19].

2.3 Biosurfactant Production Assay

The test strains were seeded on plates containing LB medium supplemented with Congo Red and containing streptomycin 100µl/ml for 24 hours at 37°C. We then inoculated the colonies in 10 ml of LB broth with appropriate antibiotics for 24 hours at 37°C in the absence of oxygen. From the 24-hour culture, we took 1 ml of the culture and inoculated it in 50 ml of LB broth with a suitable antibiotic at 37°C under agitation (250 rpm) for 24 hours until an OD between 0.6 and 0.8 was reached at 600 nm anaerobically. 1 ml of this culture was taken as stock and the rest was centrifuged at 13000 rpm. The pellet was separated from the supernatant and stored at -20°C. Positive and negative controls were grown under aerobic conditions. The ability to produce biosurfactant was done as previously described [14]. Briefly, the emulsification activity is quantified. The broth was mixed with 2 ml of gasoline, vortexed for 5 minutes, and then allowed to stand for 24 hours. By taking the layer created between the aqueous solution and the petrol layer, the height of the emulsion is

determined. The IE is determined by measuring the emulsion's size.

$$E_{24\%}=\frac{He}{Ht}\times 100$$

He = height of the emulsion; Ht = total height; $E_{24\%}$= emulsification rate after 24 hours.

2.4 Swarming Test

The ability of swarming was performed as previously described [14]. Briefly, 1 ml of a liquid overnight were inoculated in LB at 37 ° C with the addition of the selection antibiotic (streptomycinkanamycin), added it to a 50 ml flask, and then added the necessary amount of medium for 50 ml. We cultured the bacteria at 37 ° C with agitation (250 rpm) until the OD was between 0.6 and 0.7. 20 µl of the culture was placed in the middle of a Petri plate with LB + (0.5%) dextrose + (0.5%) agar, which we then incubated at 37 °C in the absence of oxygen. After observing the strain profiles for 24 hours on agar medium, we continued. We only tested positive and negative controls in an aerobic environment. Digital callipers were used to calculate the diameter of the swarms.

2.5 Anaerobically Biofilm Formation Assay

For 24 hours, we started young *Shigella* spp. cultures in SS with a *S. enterica* control. These bacteria were cultured for 48 hours at 37°C without oxygen in TSB + 1% glucose and TSB + 1% sucrose broth. These microorganisms were added to TSB broth with 1% glucose and 1% sucrose for 48 hours at 37°C without oxygen. The culture was then allowed to decant, the contents were carefully poured, and the tubes were extremely carefully washed with PBS. The tubes were then stained for 20 minutes at 37°C using 2% crystal violet or gentian violet. To remove bacteria that were only weakly adhering, the stained tubes were rinsed with distilled water before being read. The positive control was carried out under aerobic conditions and consisted of *Salmonella* sp.

2.6 Examining the Formation of Biofilms While Salicylic Acid is Present

Cultures were placed in TSB + 1% glucose + 1.25 mg/ml salicylic acid for 48 hours at 37°C without oxygen from the overnight cultures of *Shigella* strains. 2 ml of the culture were taken out and centrifuged. The supernatant obtained was used to calculate the emulsification index. Furthermore, we used 20 µl of the culture to conduct the anaerobic swarming test to evaluate the propensity of Shigella strains to move in a semisolid medium.

The remainder was decanted and the liquid was carefully poured out. After being cleaned with PBS, the tubes were stained with 2% crystal violet and 1.25 mg / ml salicylic acid, and then incubated at 37°C for 20 minutes.

2.7 The Egg Invasion Assay

A poultry egg was placed in 250 ml of LB along with 1 ml of a bacterial culture with an OD between 0.6 and 0.7. This culture was mixed and shaken for 72 hours at 37°C and 140 rpm both aerobically and anaerobically. Ethyl alcohol was used to previously sanitise the contaminated eggs. After 72 hours of contamination, the physicochemical quality of the egg contents was evaluated to determine whether the eggs were contaminated or not. The eggs were then aseptically removed from the culture medium and broken up using a heated platinum seeder after culture for 72 hours. The egg yolk was removed from the egg. Dilutions have been done and then inoculated onto SS medium before being incubated anaerobically for 24 hours. The glass vial had previously been sterilised at 121°C for 2 hours with dry heat. From the contents of the contaminated eggs, the presence of biosurfactants such as a molecule was assessed by determining the emulsification index of all samples.

2.8 Statistical Analysis

Data analysis was carried out using Excel (version 2013) and GraphPad Prism 7.

3. RESULTS

3.1 Isolation, Characterization, and Identification of *Shigella* spp.

A total of 93 strains were used in this study. 43 strains of *Shigella* spp were isolated from soil, 47 isolated from wastewater from the outskirts of Brazzaville South, and three (3) from hospital. Four (4) reference strains of *Shigella* (*S. flexneri* 5a M90T, *S. flexneri* 5a M90T, *spa40*-, *S. boydii,* and *S. sonnei*), were used as controls. The *Shigella* strains were characterized macroscopically, microscopically, and

biochemically (data not shown). All isolates with an appearance not typical of *Shigella* were not included in this study.

3.2 Molecular Identification: Restriction Fragment Lenght Polymorphism (RFLP)

Molecular Identification of isolates with a *Shigella* spp. profile on agar medium and biochemical tests were identified by 16S rDNA PCR and RFLP-PCR using the enzymes EcoRI and PstI. Agarose Gel Electrophoresis of Gene Coding PCR Products for 16S rRNA in isolates. Fig. 1 shows the electrophoresis of the 16S RNA gene RFLP-PCR products, each isolate has a DNA band of approximately 700 to 800 bp. C+ is a 16S rDNA gene without digestion (Fig. 1). All other Shigella isolates had the same electrophoretic profile.

3.3 Evaluation of Growth under Anaerobic Conditions

All strains chosen for this study demonstrated the capacity to proliferate without oxygen. The cloudiness of the culture medium, which was used to measure growth on a liquid medium (Fig. 2), was used to identify it. The range of optical density was 0.610 to 1.428. The appearance of colony-forming units on agar after 24 hours of culture at 37°C provided evidence of growth.

3.4 Exploring Biosurfactant Production

The anaerobic production of *Shigella* spp. biosurfactants bacterial strains were chosen for testing based on their capacity to emulsify hydrocarbons (gasoline) after 24 hours (Fig. 3). All strains demonstrated the ability to emulsify hydrocarbons with index values ranging from 30% to 100% in anaerobic conditions (Fig. 3A). Gasoline emulsion formation was only inhibited by the *S. flexneri* 5a M90T mutant (Fig. 3A et B).

3.5 Swarming Test

The ability of *Shigella* spp. strains to swarm on a semisolid medium was evaluated. This study showed that except for *S. flexneri* 5a M90T *spa40-*, all (100%) of the *Shigella* spp strains tested had the ability to swarm on semisolid medium with swarming diameters between 75 and 80 cm on Petri dishes after 24 h (Fig. 4).

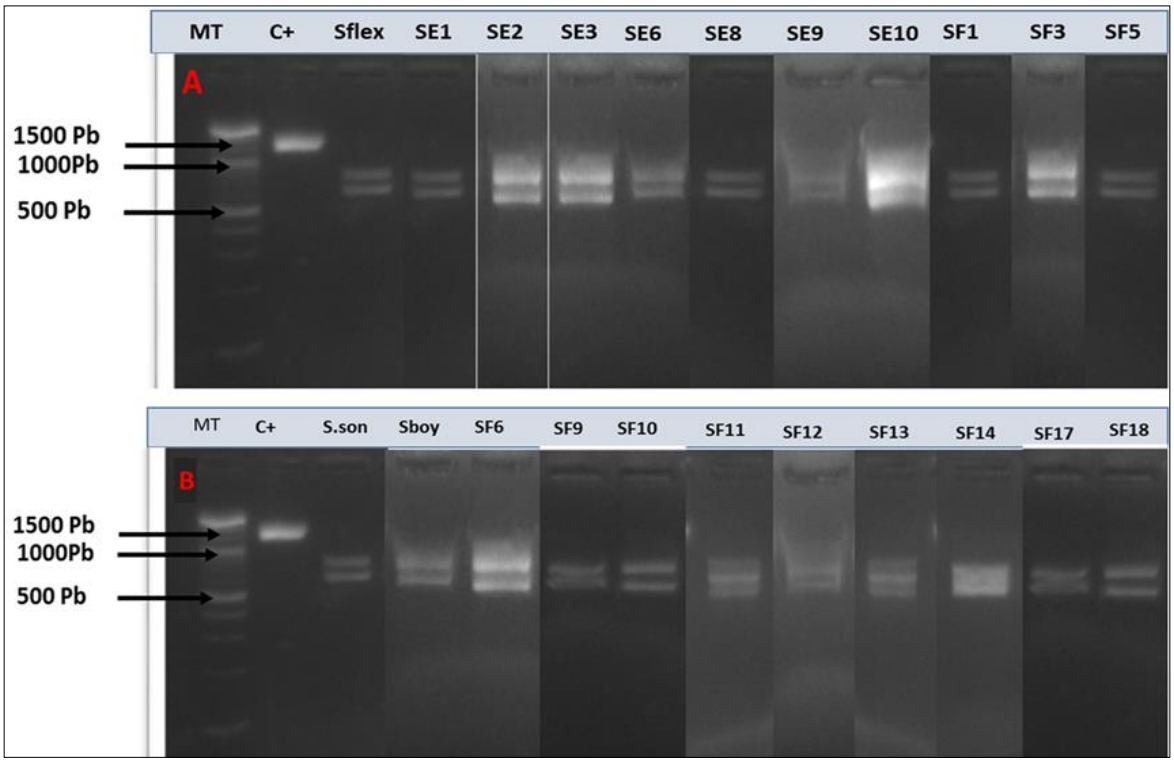


Fig. 1. Enzymatic digestion profile of 16S rRNA gene PCR products. Legend MT= 2log size marker; Sflex: *S. flexneri* 5a M90T; Sson: *S. sonnei*; Sboy: *S. boydii*; SE: environmental *Shigella* strains isolated from water; SF: environmental *Shigella* strains isolated from faecal contaminated soil

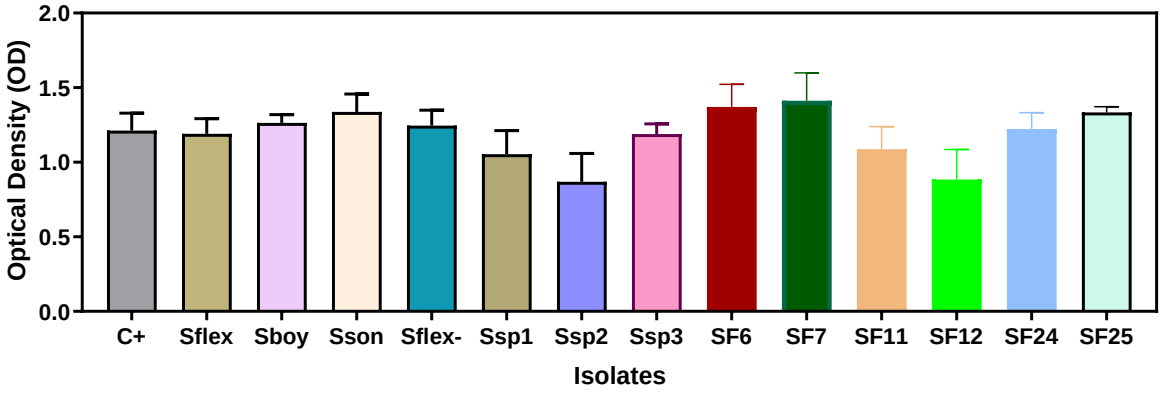
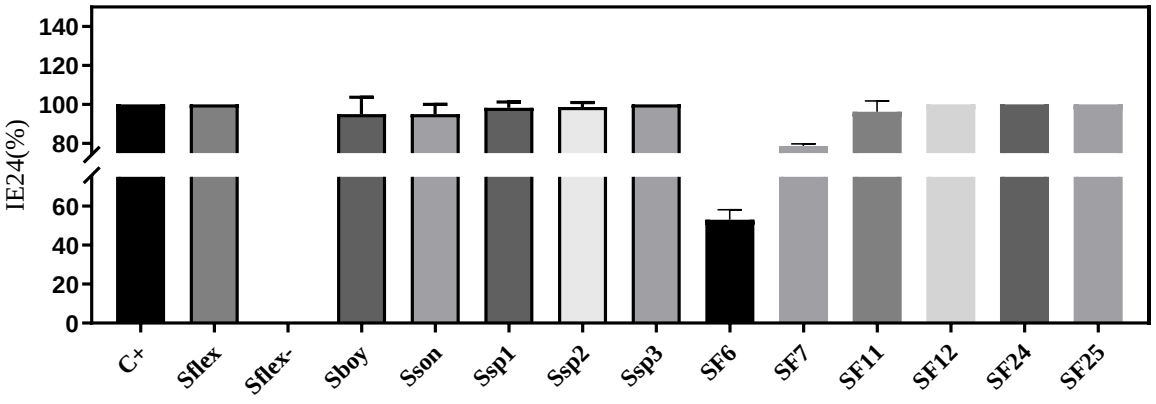


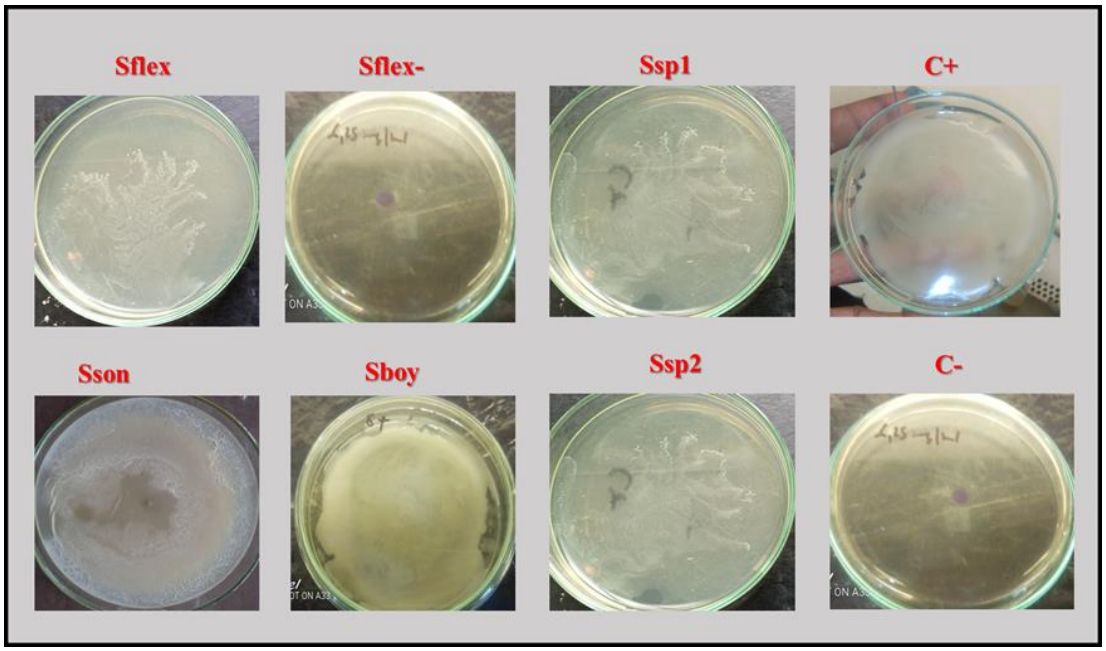
Fig. 2. Croissance de quelques souches de *Shigella* spp en anaérobiose. C+ : *Salmonella* strain, Sflex : S. flexneri, Sboy : S. boydii, Sflex- : S. flexneri mutant, Ssp1, 2 and 3: Clinical strains. SF6, 7, 11, 12, 24 and 26: Environmental strains



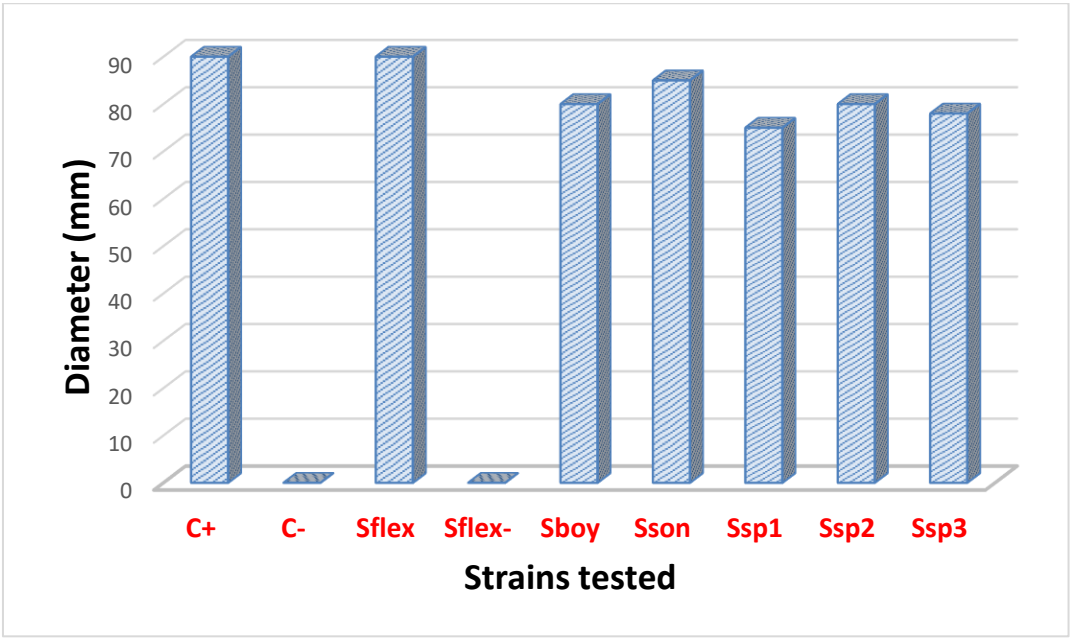
(a)

(b)

Fig. 3. Emulsification test of some Shigella spp. strains under anaerobic conditions (a). Emulsification profile of some tested strains; (b) appearance of the different strains tested after 24 hours. Legend: C+: *S. flexneri* 5a M90T grown aerobically; C-: negative control (*S. flexneri* 5a M90T *spa* 40-); Sflex: *S. flexneri* 5a M90T, Sflex: *S. flexneri* 5a M90T *spa* 40-; Sson: *S. sonnei*; Sboy: *S. boydii*; Ssp1-3: hospital *Shigella* sp. SF6, 7, 11, 12, 24 and 26: Environmental strains



(a)

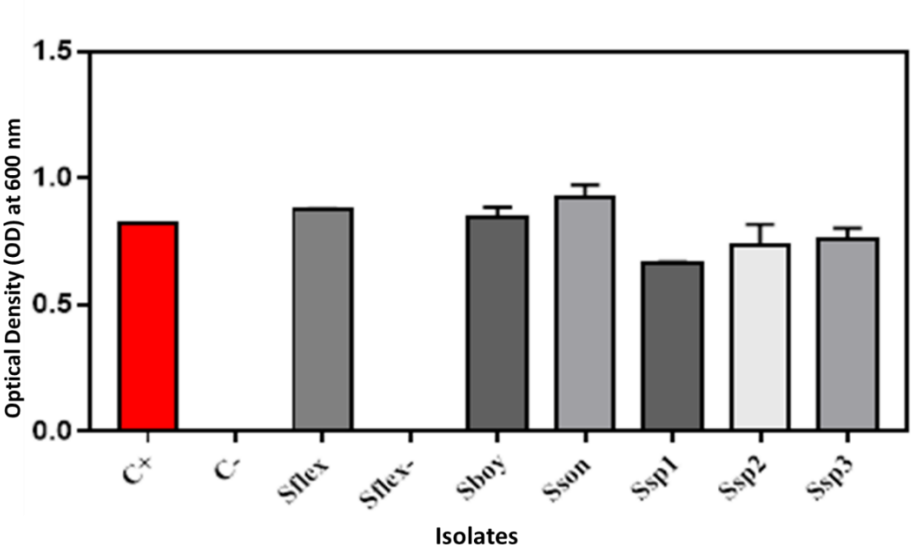


(b)

Fig. 4. Swarming test of some strains of *Shigella* spp. in anaerobic conditions after 24 h (a). Swarming profile of some *Shigella* strains on semisolid medium; (b). Swarming diameter values of some Shigella spp. strains on soft agar after 24h. Legend: C+: S. flexneri 5a M90T grown aerobically; C-: negative control (S. flexneri 5a M90T spa 40-); Sflex: *S. flexneri* 5a M90T, Sflex: *S. flexneri* 5a M90T spa 40-; Sson: *S. sonnei*; Sboy: *S. boydii*; Ssp1-3: hospital *Shigella* sp.

3.6 *Shigella* and Biofilm Formation

The *S. flexneri* 5a M90T *spa40* mutant was the only *Shigella* spp. strain that was unable to form biofilms after 24 hours, according to the crystal violet adhesion test. Both aerobic (positive control) and anaerobic conditions caused the strains to form biofilms. The optical density values for biofilm formation ranged from 0.6 to 1.2 (Fig. 5).



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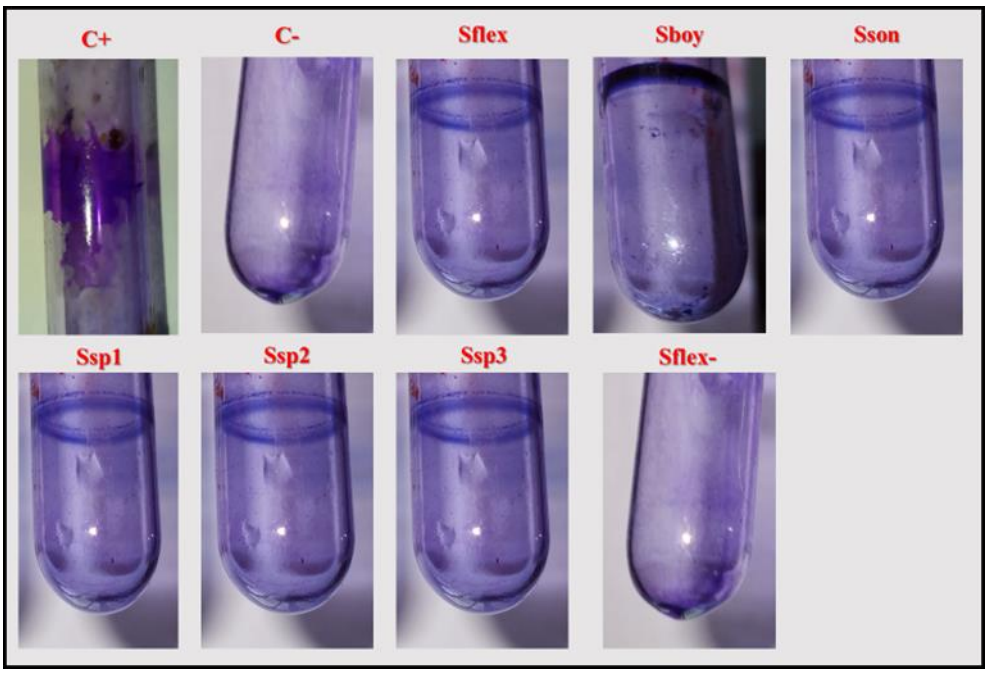


(b)

Fig. 5. Biofilm formation test by some strains of Shigella spp. used in this study (a). Values of the corresponding optical densities of the cultures tested; (b). Highlighting of biofilms formed on the walls of the tubes. Legend: C+: *S. flexneri* 5a M90T grown aerobically; C-: negative control (*S. flexneri* 5a M90T *spa* 40-); Sflex: *S. flexneri* 5a M90T, Sflex: *S. flexneri* 5a M90T *spa* 40-; Sson: *S. sonnei*; Sboy: *S. boydii*; Ssp1-3: hospital *Shigella* sp.

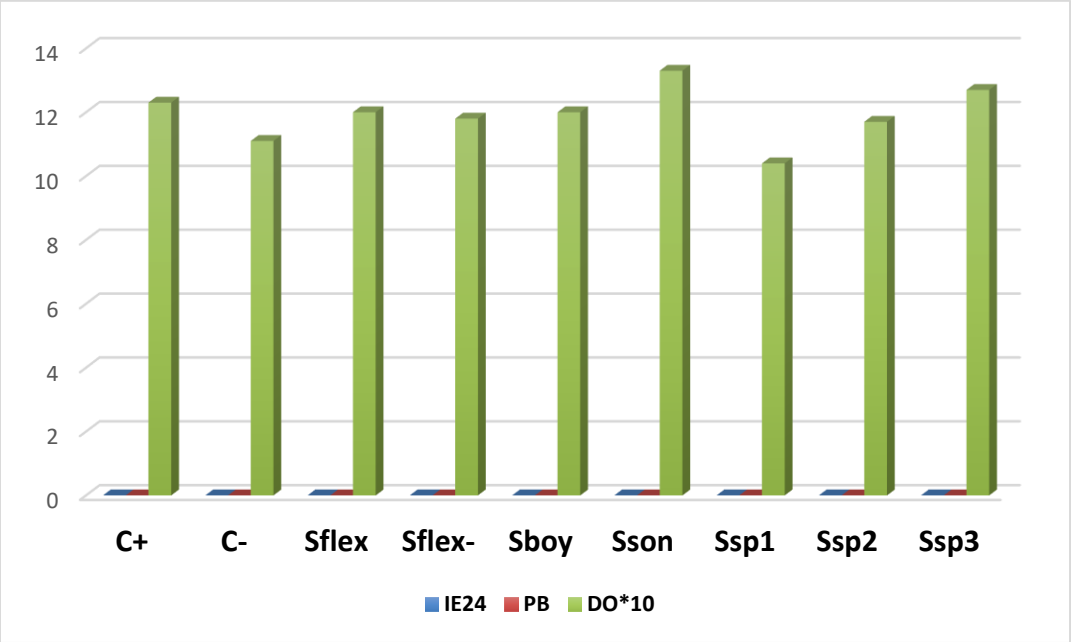
3.7 Correlation between Biosurfactant Production and Biofilm Formation in *Shigella* spp.

The strains that could form biofilms were also able to emulsify hydrocarbons in anaerobic conditions, according to the monitoring of biosurfactant secretion and biofilm formation (Fig. 6).

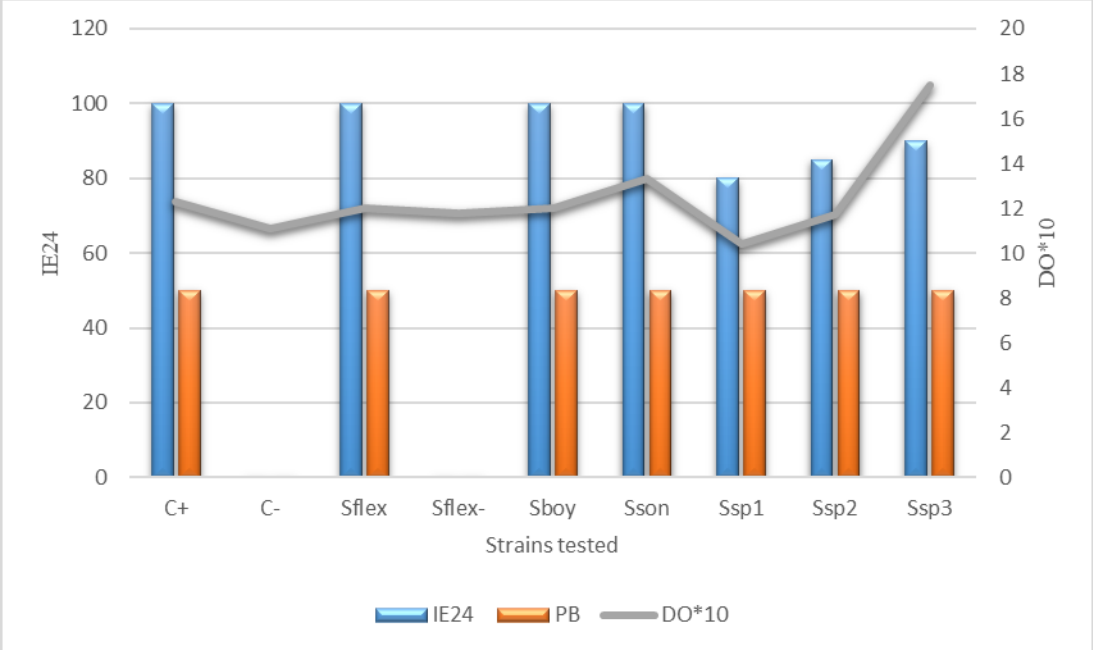
3.8 Egg Contamination Assay

By assessing the physicochemical degeneration of the egg contents, the test for *Shigella* spp.

contamination of eggs was determined. Changes in the texture, color, and odor of the contaminated egg contents, as well as the decoagulation or liquefaction of the yolk (Fig. 7). This experiment demonstrated that under anaerobic conditions, none of the Shigella strains could contaminate the eggs. The only strain that could invade the egg in an aerobic test was the control strain (Fig. 7).



(a)



(b)

Fig. 6. Relationship between optical density, the formation of biofilms, and the release of biosurfactants (a) of salicylic acid is present. (b) when salicylic acid is absent. Legend: C+: *S. flexneri* 5a M90T grown aerobically; C-: negative control (*S. flexneri* 5a M90T *spa40*-); Sflex*: S. flexneri* 5a M90T, Sflex: *S. flexneri* 5a M90T *spa* 40-; Sson: *S. sonnei*; Sboy: *S. boydii*; Ssp1-3: hospital *Shigella* sp.

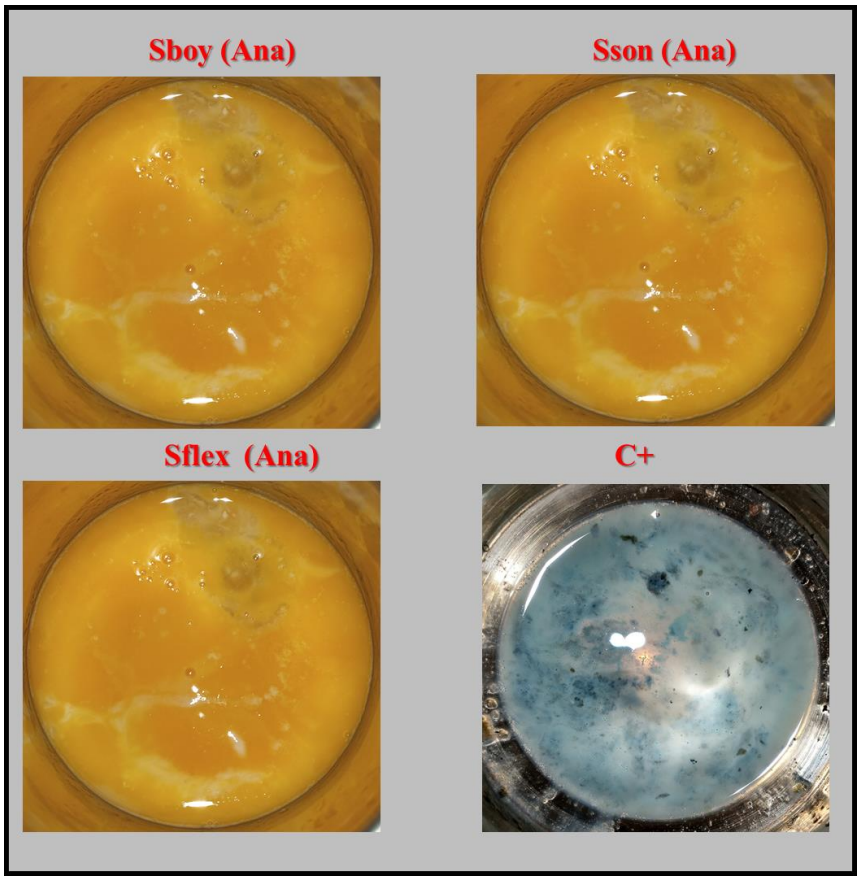


Fig. 7. Appearance of the egg's contents following the test for anaerobic contamination. Legend: C+: *S. flexneri* 5a M90T grown aerobically; Sflex: *S. flexneri* 5a M90T; Sson: *S. sonnei*; Sboy: *S. boydii*; Ssp1-3: hospital *Shigella* sp.

3.9 Bacteria Counting after Egg's Contamination by *Shigella* Strain

Under anaerobic conditions, no strain was able to spread infection to the eggs. With an invasion rate of 39.13%, the egg was only capable of being invaded by the aerobically grown positive control. The outcomes are shown in Table 1.

4. DISCUSSION

This research was carried out to advance the understanding of the mechanism by which *Shigella* spp. invades epithelial cells. The aim of this paper is to describe specifically how *Shigella genus* bacteria behave when producing and using biosurfactants as a virulence factor in the colonisation of epithelial cells under anaerobic conditions. In this study, 93 different *Shigella* spp. isolates, including four (04) reference strains, and three (3) strains found by genus-based identification (biochemical reaction and culture characters in boxes), were used in this study. Our study established that the strains used in this study belonged to the genus *Shigella*. This has been previously demonstrated [17]. Antibiotics were used to choose and phenotypically confirm purified *Shigella* strains [14]. All isolates were detected to be members of *Shigella* spp. since the group had restriction fragment profiles that matched those of the reference strains that had previously been molecularly identified.

Table 1. *Shigella* species' invasiveness when exposed to anaerobic environments

Strain	C+	C-	Sflex	Sboy	Sson	Sflex-	Ssp1	Ssp2	Ssp2
Before	+	+	+	+	+	+	+	+	+
After	+	-	-	-	-	-	-	-	-

Before: before invasion; After: after invasion; +: positive; - :negative

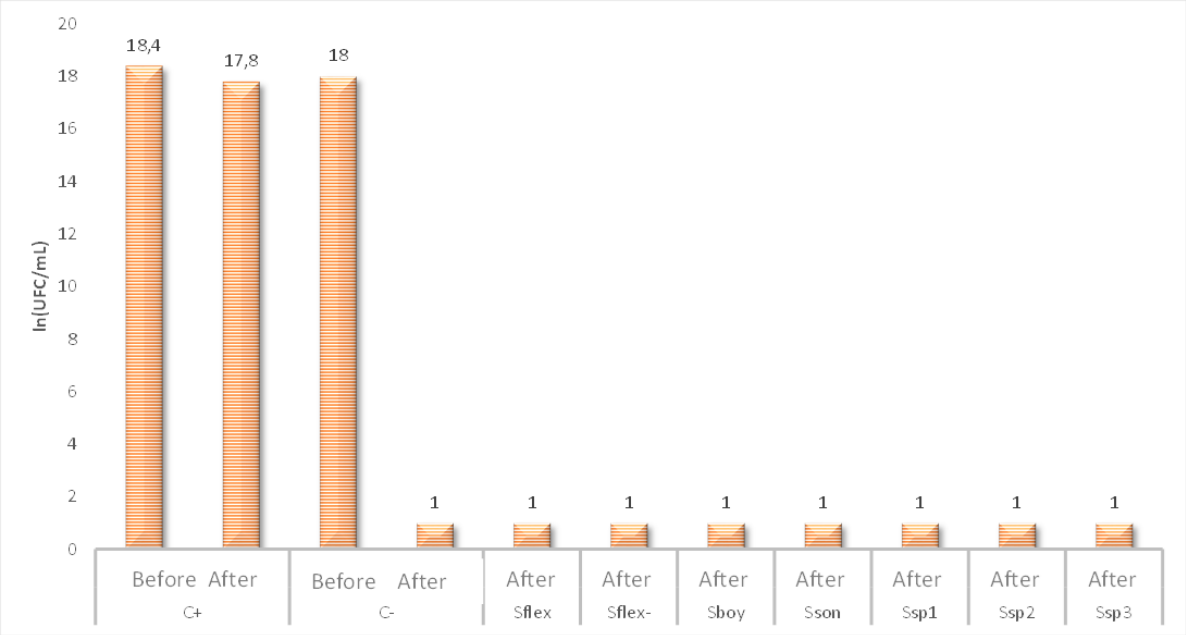


Fig. 8. *Shigella* spp. strains' invasiveness in anaerobic environments. Legend: C+: *S. flexneri* 5a M90T grown aerobically; C-: negative control (*S. flexneri* 5a M90T spa 40-); Sflex: *S. flexneri* 5a M90T, Sflex: *S. flexneri* 5a M90T spa 40-; Sson: *S. sonnei*; Sboy: *S. boydii*; Ssp1-3: hospital *Shigella* sp.

The Shigella spp. strains used in this study have all demonstrated the ability to grow with and without oxygen [20]. Many environmental signals and regulatory systems are integrated into oxygen-dependent pathways. SRNAs, FnrS, and ArcZ, which repress hilD translation, leading to decreased HilA production. FnrS and ArcZ are oppositely regulated in response to oxygen, one of the key environmental signals that affect SPI1 expression of SPI1 [20]. Since Shigella is a facultative aeroanaerobic bacterium, some effector proteins can be expressed in both cases [19]. *E. coli* and *Salmonella* show that FnrS is produced under anaerobic conditions due to Fnr-mediated activation of fnrS transcription, while ArcZ is produced mostly beneath aerobically; its transcription is repressed anaerobically by ArcA-P [19,21,22]. Our findings support *Shigella's* facultative anaerobic phenotype, which is a type of bacteria. *Shigella* can grow in an anaerobic environment because this setting mimics that of the colonic lumen, where they are the most pathogenic, and the colonic epithelium [23].

The emulsification test is an indirect test that demonstrates the production of substances with surface-active properties in the extracellular medium [24-27]. The ability of specific bacteria to secrete biosurfactants can be highlighted through the emulsification test [28]**.** Our previous data showed that *Shigella* is able to produce and secrete biosurfactants when there was oxygen leakage and T3SS was being induced. The same study claims that these biomolecules may be crucial for the pathogenesis of *Shigella* [14]. The attachment of bacteria to the surface is facilitated by biosurfactants, which are found in bacteria such as *Shigella* and *Salmonella enteridis Typhimurium* SE 86, which belong to the same family [29]**.** The fact that biosurfactant is a Shigella virulence factor secreted under anaerobic conditions demonstrates that bacteria of the genus Shigella can escape cell surveillance by using anaerobic pathway machinery. Biosurfactants are compounds that have the ability to emulsify hydrocarbons [28,30].

Biosurfactants have been implicated in a number of multicellular phenomena in *P. aeruginosa*, including the formation of biofilms and the movement of swarms in semisolid media [28,31].

The formation of biofilms is an asset for bacteria of the genus Shigella in the propagation of their pathogenicity. Furthermore, the swarming of *Pseudomonas aeruginosa* has been described to *Proteus mirabilis* [31], *Salmonella* sp. [32], and *Shigella* [33]. This study demonstrated that all strains of *Shigella* spp. tested could swarm on semisolid media under anaerobically. This finding

demonstrates that *Shigella* can swarm with and without oxygen. This finding demonstrates that, regardless of the environment or stage of infection, *Shigella* spp. produce and secrete biosurfactants in the extracellular environment. In fact, a link has been found between a strain's ability to produce biosurfactants and its propensity to swarm in *P. aeruginosa* [34,35].

Additionally, it was established that the bacterial flagellum is not necessary for the swarming phenotype. This study supports the findings of the previous investigation conducted in 2020 by Kinavouidi et al. [14]. According to this study, *Shigella* spp. produces and secretes biosurfactants as part of its growth process or co-translation. Under these circumstances, the biosurfactant of *Shigella* spp. is a virulence factor involved in surface or membrane attachment processes (role of adhesin), as previously demonstrated in *P. aeruginosa* or *Salmonella* sp. [34,35], and would give *Shigella* spp. cytolytic power in addition to the effectors typically described for T3SS. It is possible that the biosurfactant alters the colonic epithelium and alters cell function related to mucosal immunity.

Therefore, this study supports the finding that Shigella spp. can form biofilms without oxygen. The biofilms of these bacterial species are created in part by P. aeruginosa. In light of the findings of this study, we believe that *Shigella* biosurfactant would perform the same invasion function. In fact, not every factor that causes *Shigella* spp. to form biofilms has been clearly identified. *The capacity of Shigella* spp. to produce and secrete biosurfactants has not been previously studied, so a cause-and-effect connection could not be proven. However, this study demonstrated that the number of bacteria present is necessary for biofilm formation. This study also suggests the same conclusion regarding the propensity of strains to swarm and emulsify hydrocarbons. Consequently, there is a correlation between these three constants: the optical density that characterises the amount of bacterial biomass present, the ability of strains to secrete biosurfactants, and the ability of strains to form biofilms. The ability of *Shigella* to form biofilms was described by Kourtney and colleagues [33,36]. By arguing that *Shigella* biosurfactants have a crucial role to play, the current study supports the findings of Kourtney and colleagues.

Shigella spp. strains failed the chicken egg contamination test to mimic invasion, showing that they cannot enter embryonic yolk cells. In fact, none of the strains tested showed any growth in the culture medium after 24 hours of anaerobic conditions at 37 ° C. The results of the emulsification test conducted on egg content were negative, demonstrating the lack of any surfactant inherent to the egg itself or attributable to the resident microbial flora of the egg. Only the aerobic control, or with oxygen present, was successful and allowed the isolation of colony-forming units in particular media after 24 hours. This finding emphasises the lack of cell invasion by *Shigella* spp. strains and the crucial role that oxygen plays in triggering invasion. The reason for this is that the outcome obtained in the absence of oxygen differs from the outcome obtained in the presence of oxygen.

Shigella spp. have a protein machinery called T3SS, so the fact that none of the *Shigella* strains invaded in this study is due to the ability to function. T3SS is oxygen dependent, as demonstrated by recent research by Benoît Marteyn et al. Without oxygen, T3SS is inhibited by a factor called FNR (fumarate-nitrate reductase), which in turn inhibits Spa33 and Spa32 [19]. Spa33 is one of subunits of the Spa47 ATPase responsible for the production of the proton motive force that provides energy for the translocation of effectors from the bacterial cytosol to the cytoplasm of the host cell [19].

Spa32 is a T3SS molecule that acts as a molecular ruler and determines the size of the needle, a sine qua non condition for good T3SS activity. In fact, Botteaux et al. in 2008 demonstrated that *Shigella* with a larger or shorter size than normal size was unable to invade [37]. We also think that the findings of Botteaux et al. may help explain why the tested bacterial cells were unable to invade or contaminate eggs, as a result of Spa33 and Spa32 being simultaneously inactive and repressed by the FNR factor (T3SS regulator). Our findings are consistent with those of Benoit Marteyn and associates, who also showed that *Shigella* spp. could not colonise epithelial cells [19].

Nuanced findings regarding the strains' capacity to invade cells in the presence or absence of oxygen support the conclusions made by Kinavouidi et al. regarding the pathways by which *Shigella* secretes biosurfactants. As shown by Kinavouidi et al. [33], *Shigella* has developed other adaptability mechanisms in epithelial cell

invasion mediated by the secretion of biosurfactants.

Indirectly, this work raises the question of the chemical nature of the biosurfactant secreted by *Shigella* spp. Given its secretion through T3SS, the biosurfactant must be totally or partially proteinaceous as suggested by Kinavouidi et al. [14].

The inhibition of Spa33 and Spa32 in *Shigella* spp. helps explain the leakage of T3SS because, as described by Claude Parsot in 2009 [23], T3SS is activated simultaneously by contact with the host epithelial cell. Due to the villi rich in blood vessels, the residual oxygen diffusing from the capillaries allows inhibition of T3SS to be lifted by diffusion of oxygen within the bacterial cell [19]. Indeed, in the absence of oxygen and consequently in the absence of cell contact, T3SS is partially assembled and consequently cannot allow the escape of certain effectors, among them the biosurfactant. In fact, this result is correlated with the phenotype of the Spa40 mutant.

5. CONCLUSION

The present work has shown that: (i) *Shigella* bacteria are capable of producing and secreting biosurfactants in extracellular medium under anaerobic conditions; (ii) *Shigella* bacteria are capable of swarming in semisolid medium under anaerobic conditions; (iii) *Shigella* bacteria are capable of forming biofilms under anaerobic conditions; (iv) Biosurfactants produced by Shigella are considered involved in the formation in *Shigella* spp; (v) there is a correlation between bacterial concentration, the ability to secrete biosurfactants and the ability of strains to form biofilms; (vi) *Shigella* bacteria are unable to invade cells under anaerobic conditions; (vii) secretion of biosurfactants in the absence of oxygen occurs under leaky T3SS conditions.

DISCLAIMER (ARTIFICIAL INTELLIGENCE)

Author(s) hereby declare that NO generative AI technologies such as Large Language Models (ChatGPT, COPILOT, etc.) and text-to-image generators have been used during the writing or editing of this manuscript.

DATA AVAILABILITY

The Excel sheets including the data used to support the findings of this study are available from the corresponding author upon request.

CONSENT

It is not applicable.

ETHICAL APPROVAL

It is not applicable.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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Peer-review history:
The peer review history for this paper can be accessed here:
https://www.sdiarticle5.com/review-history/127940