Profiling the first production assessment of *Shigella* biosurfactants like moleculein anaerobic conditions

Abstract

The mechanisms understanding of pathogenicity of enteric bacteria such as *Shigella* is important in the design of new therapeutic targets for the optimal management of these infections. This study on ninety-three (93) *Shigella* strains (*Shigellaflexneri* 5a M90T, *Shigellaflexneri* 5a M90T *spa40*-a , *Shigella boydii*, *Shigella sonnei*, and three *Shigella* spp.) found that all *Shigella* strains (n = 93) except the mutant of *Shigella flexneri* 5a M90T are able to emulsify hydrocarbons with index these strains also swarmed on semi-solid 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 (anaerobiosis). The invasion rate was zero in anaerobiosis and over 50% in aerobiosis. These results show that biosurfactants are produced in a cotranslational pathways and are secreted into the extracellular medium via *Shigella* SST3. *Shigella* biosurfactants are involved in biofilm formation and are secreted into the extracellular medium via the phenomenon known as "SST3 leakage."

Key words: *Shigella*, pathogenicity, anaerobiosis, biosurfactant, SST3.

Introduction

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

Shigella spp. are Gram-negative, strictly pathogenic, facultatively aero-anaerobic, immobile, non-encapsulated, 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 slowly ferment **[2]**. 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 diarrhea. Tropical countriesincluding Republic of Congo continue to experience this infection, especially during the warmer and colder seasons**[3, 4]**.

In addition to the lipopolysaccharides on the outer membrane of *Shigella* spp., other components involved in pathogenicity include adhesins, invasins, toxins, protein secretion system, and iron absorption system. **[4]**. *Shigella* spp. are therefore entero-invasive bacteria, capable of penetrating the epithelial cells of the intestinal mucosa and multiplying there, leading to the formation of abscesses and ulcerations. These bacteria do not enter the general circulation, but local phenomena may be accompanied by toxicosis and dehydration **[5].** The invasive phenomenon affects the superficial mucosa and decreases to the deeper layers. These germs can cause widespread epidemics or sporadic cases and are only found in humans **[4]**.

In terms of global epidemiology, *Shigella* is the second most common cause of rotavirus-related diarrheal disease death in children under the age of five. In actuality, it results in nearly 164300 fatalities annually throughout the world, 54900 of which occur in sub-Saharan Africa and South Asia **[6]**. There are no epidemiological data on this illness available for the Republic of Congo.

Shigella is able to invade under anaerobic conditions and form biofilms, which in some circumstances allows it to cross the digestive tract and reach the colon where the infection occurs **[7]**. This pathogenicity profile is defined by its type 3 secretion apparatus, which allows the delivery of virulence effectors into the host epithelial cell.

According to the most recent findings of ongoing clinical trials, there hasn't 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... **[8].**

It has recently been demonstrated that all *Shigella* spp. strains isolated from the environment, including *Shigella flexneri*, *Shigella boydii*, *Shigella sonnei*, and others, have the capacity to produce and secrete biosurfactants into the extracellular medium. The same study demonstrated that biosurfactant was secreted via the type 3 secretion system under leaky and induced conditions **[9]**.

The type 3 secretion system is also demonstrated to be oxygen-dependent. The type 3 secretion system does not function in anaerobic environments. In these circumstances, *Shigella* is unable to invade and deliver virulence effectors into the host epithelial cell. However, the ability to produce biosurfactants in preinvasion and proinvasion situations has only been tested under aerobic conditions. Additionally, the degree of antibiotic tolerance was not investigated.

This research contributes to our understanding of the invasion mechanism of epithelial cells by bacteria of the genus *Shigella*. By investigating these bacteria's ability to produce and secrete biosurfactants in the extracellular medium under anoxic conditions, we hope to complete the work started by Kinavouidi and colleagues in 2020.

Methods

Strains and Culture Conditions

For environmental strains, five (5) main collection sites, and for clinical strains, one site, isolates were gathered. In total, we collected three (3) clinical strains, forty-three (43) isolates from faecal-contaminated soils, and forty-seven (47) isolates from wastewater. Four (4) *Shigella* laboratory strains (S. *flexneri*, *S. flexneri* Spa40-, *S. sonnei*, and *S. boydii*) were used as reference strains. Petri dishes were incubated at 37˚C for 24 h. After the first isolation on Petri dishes, different colonies were obtained. Each *Shigella's* characteristic colony from SS was separately isolated. Purification of the isolates was rigorously done by successive and alternating subcultures. Purity was estimated by using a microscope for morphological characterization. Gram status was determined by using 3% KOH.

Identification of Isolate and Genomic DNA Extraction and RFLP-PCR

To confirm *Shigella* isolate, genomic DNA extraction and purification was performed using NucleoSpin Microbial DNA kit (Macherey-NAGEL). Briefly, the targeted isolate were 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 ratio of optical density 260/280 nm. The housekeeping 16S rRNA gene has been amplified by PCR (Thermal Cycler, Bio-Rad) by 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). 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 solution of Gel Extraction kit (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 demonstrated that the isolated strains are members of a particular bacterial genus [20].

Study of the pathogenicity of *Shigella* strains

Screening of biosurfactant production

Preparation of *Shigella* spp. for culture under anaerobic conditions

The test strains were plated on plates containing LB medium supplemented with Congo Red and containing the appropriate antibiotics 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 into 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 under anaerobic conditions. 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. The positive and negative controls were grown under aerobic conditions.

Determination of the emulsification index

By calculating the Emulsification Index (EI24) for 24 hours, the emulsification activity is quantified. The broth were mixed with 2 mL of gasoline (available at gas stations), 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. IE is determined by measuring the emulsion's size.

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

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

Test de Swarming

We extracted 1 mL from an overnight culture in liquid LB at 37°C with the addition of the selection antibiotic, 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. We took 20 µL of the culture and put it in the middle of a Petri dish with LB + (0.5%) dextrose + (0.5%) agar, which we then incubated at 37 °C in the absence of oxygen. After observing the strains' profiles for 24 hours on agar medium, we continued. We only tested positive and negative controls in an aerobic environment. Digital calipers were used to calculate the diameter of the swarms.

Test of biofilm formation in anaerobic environments

For 24 hours, we started young *Shigella* spp. cultures on SS with a *Salmonellaenterica* 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 get rid of bacteria that were only weakly adhering, the stained tubes were rinsed with distilled water before being read. The positive control was performed under aerobic conditions and consisted of Salmonella sp.

Examining the formation of biofilms while salicylic acid is present

The cultures were plated on TSB + 1% glucose + 1.25 mg/mL salicylic acid for 48 hours at 37 °C without oxygen from the overnight cultures of the tested *Shigella* strains. 2 mL of the culture were taken out and centrifuged. The obtained supernatant was used to calculate the emulsification index. Additionally, we used 20 µL of the culture to conduct the anaerobic swarming test to evaluate the Shigella strains' propensity for moving on 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 1.25mg/mL of salicylic acid, and then incubated at 37°C for 20 minutes.

Test for anaerobic invasion or egg contamination

***Shigella* strain culture for contamination**

A poultry egg was placed in 250 mL of LB along with 1 mL of an overnight bacterial culture with an OD between 0.6 and 0.7. This culture was shaken for 72 hours at 37 °C and 140 rpm both aerobically and anaerobically. Ethyl alcohol was used to previously sanitize the contaminated eggs. After 72 hours of contamination, the physico-chemical quality of the egg contents was evaluated to determine whether the eggs were contaminated.

Egg content counting of bacteria

The eggs were aseptically removed from the culture medium and broken up using a heated platinum seeder after being cultured for 72 hours. The egg yolk was removed from the egg and inoculated onto the SS medium before being incubated anaerobically for 24 hours. The glass vial had previously been sterilized at 121°C for 2 hours with dry heat.

From the contents of the contaminated eggs, the presence of biosurfactants was assessed by determining the emulsification index of all samples.

Statistical analysis

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

Results

Sampling

In order to sample wastewater, faecal-contaminated soil, and water sources, four Brazzaville le Pool districts—I Makélékélé, II Bacongo, III Poto-Poto, and VIII Madibou—were chosen. Three samples were taken per site from each of the eleven sampling points that were chosen (Figure 1).

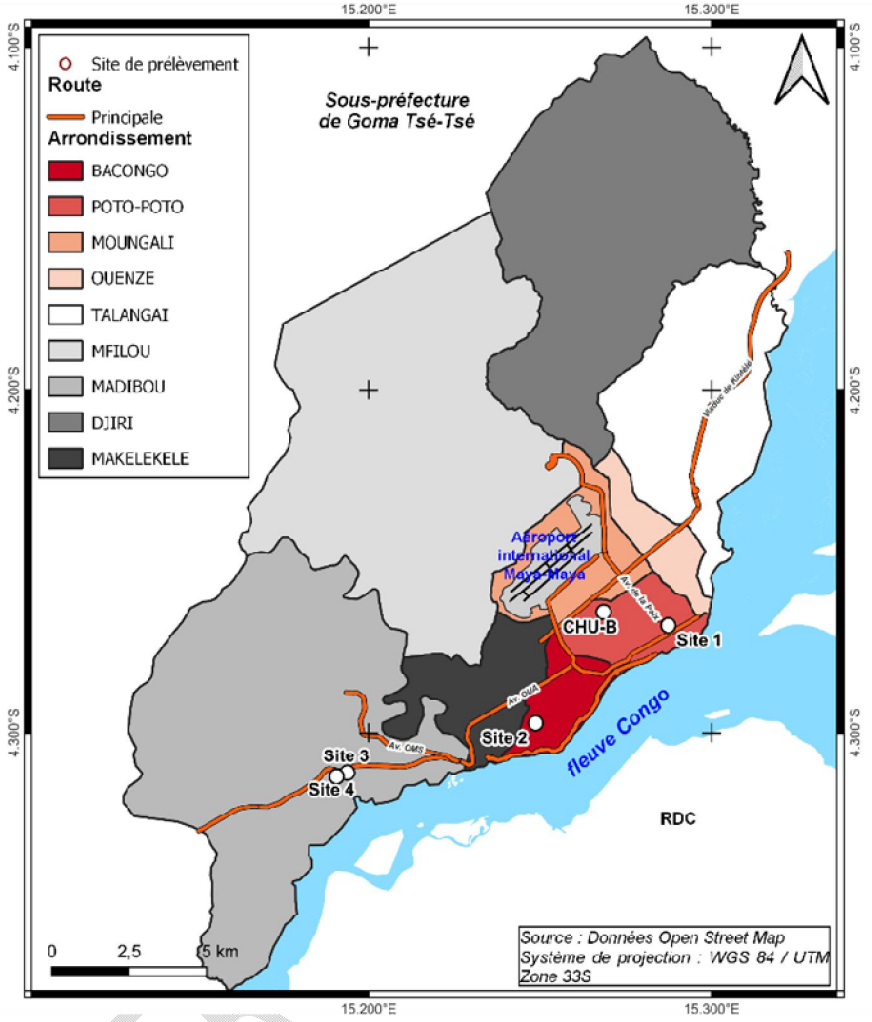


Figure 1 : Location of sample collection and analysis for Shigella spp. isolates.

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. These strains were isolated from Hektoen medium and purified on SS medium. 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.

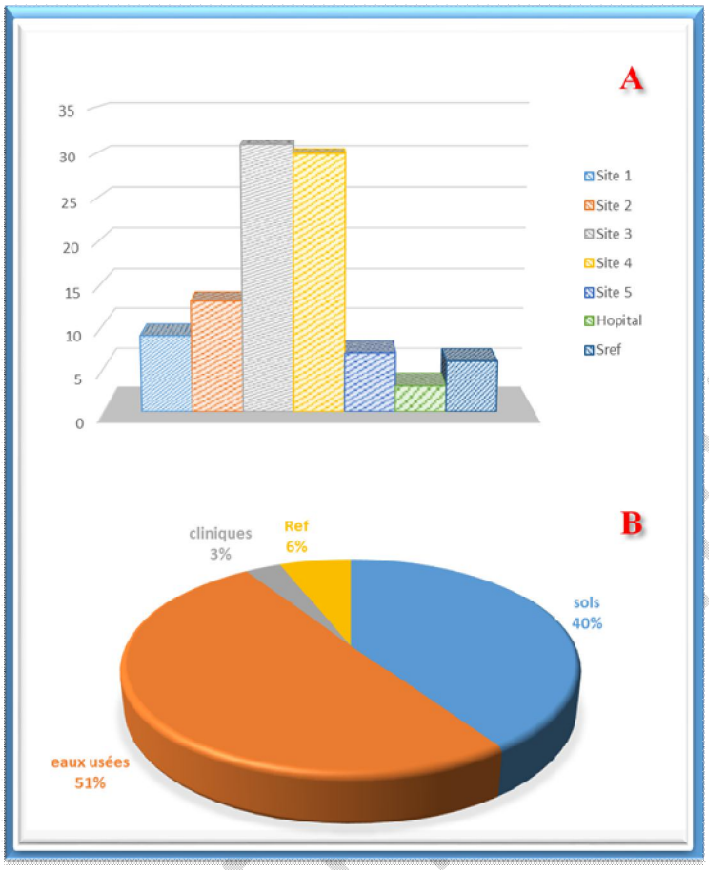


Figure 2:Origin of the isolates used in this study. A. Number of isolates per collection site; B. Distribution by sample type.

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. Figure 4 shows the electrophoresis of the 16S gene RFLP-PCR products, each isolate has a DNA band of approximately 700 to 800 bp. C+ is a 16S rDNA gene without digestion (**Figure 3**).

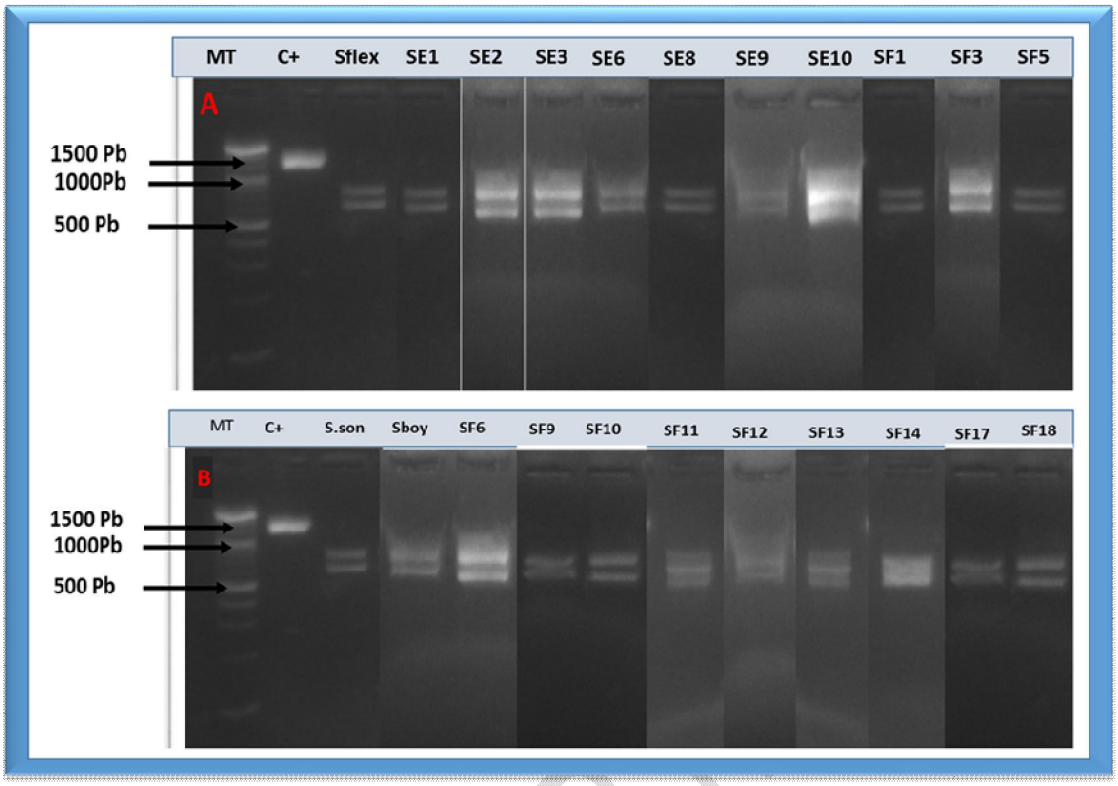


Figure 3:Enzymatic digestion profile of 16S rRNA gene PCR products.

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.

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 (Figure 4), 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.

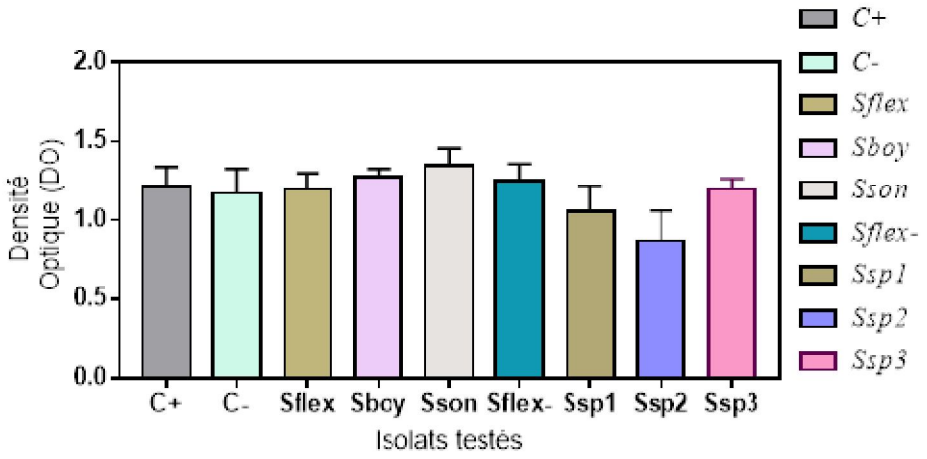
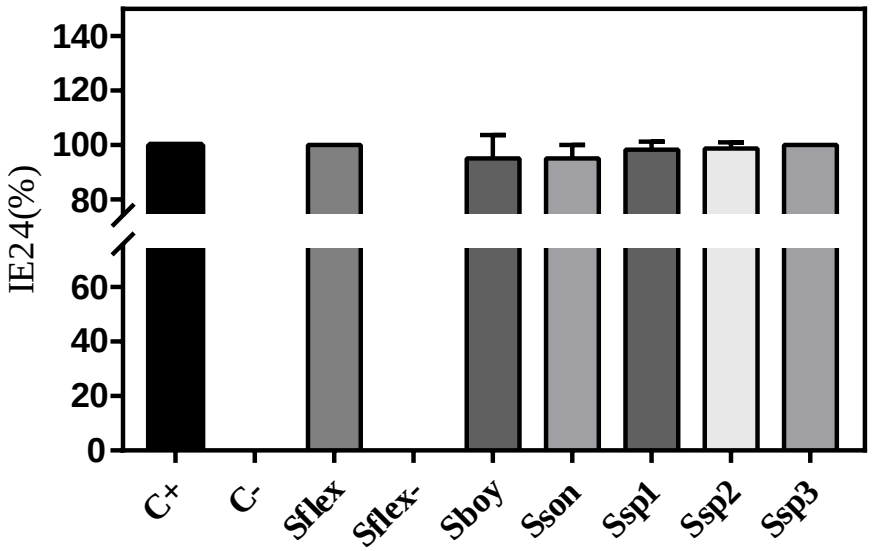


Figure 4 : Croissance de quelques souches de *Shigella* spp en anaérobiose.

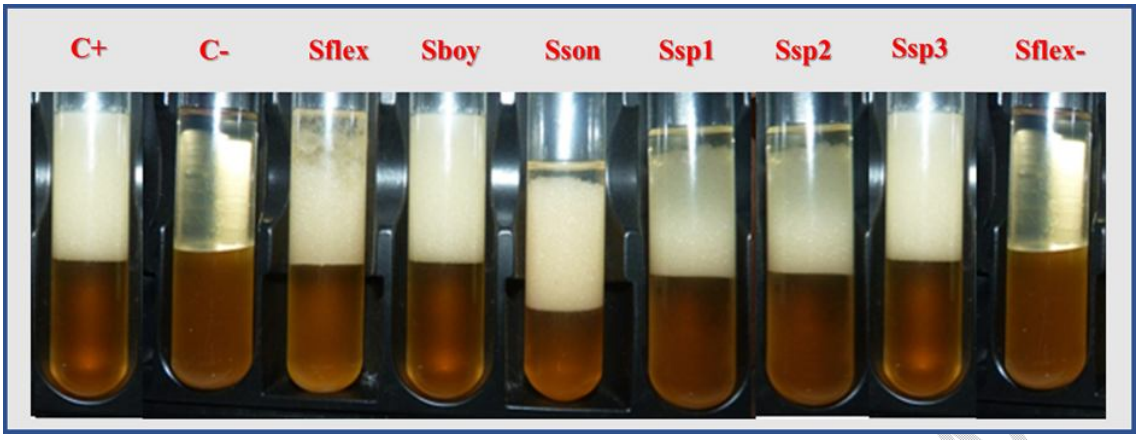
Biosurfactant production assay

Emulsification test

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



(a)



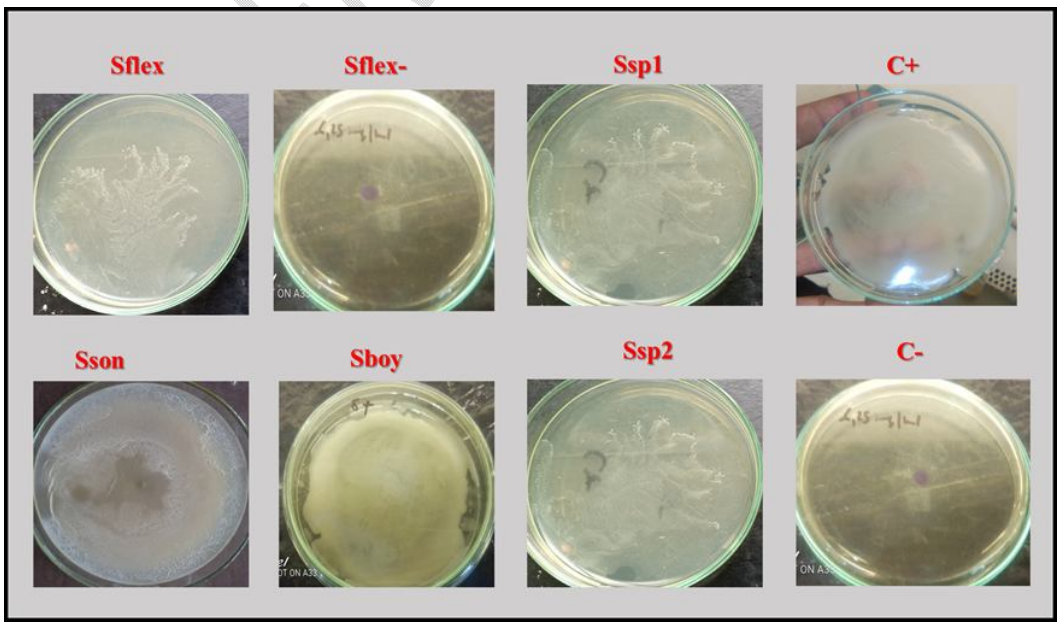
(b)

Figure 5: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.

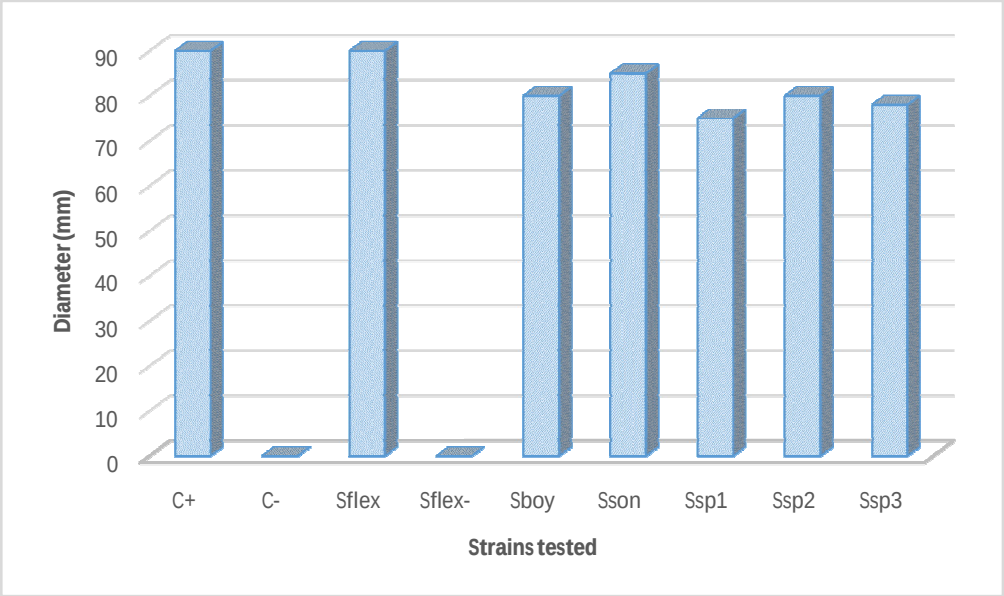
Legend: C+: *S. flexneri* 5a M90T grown aerobically; C-: negative control (*S. flexneri* 5a M90T *spa* 40-); Sflex: *S. flexneri* 5a M90T, Sflex: *S. flexneri* 5a M90T *spa* 40-; Sson: *S. sonnei*; Sboy: *S. boydii*; Ssp1-3: hospital *Shigella* sp.

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 (**Figure 6**).



(a)



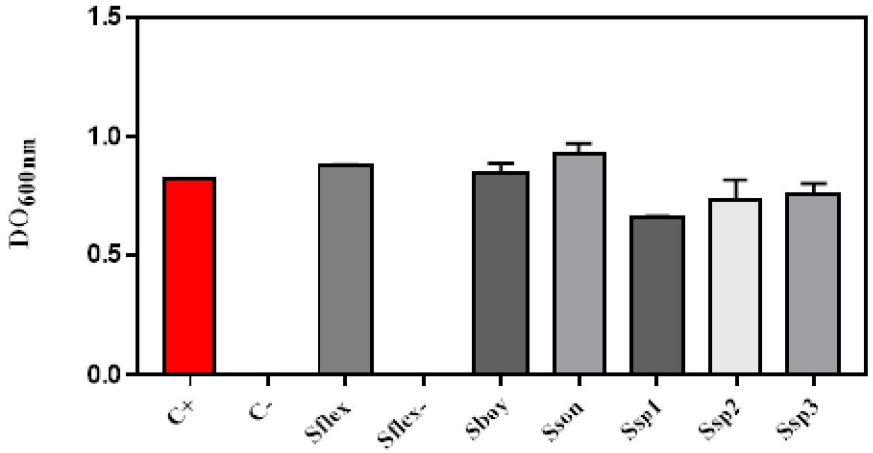
(b)

Figure 6 : 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.

Biofilm formation assay

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 (**Figure 7**).



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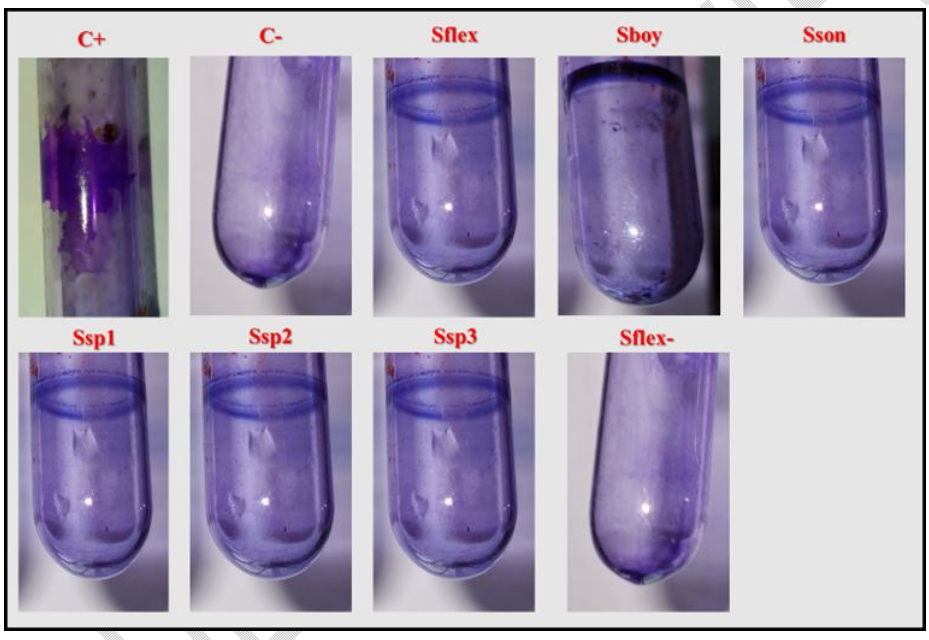


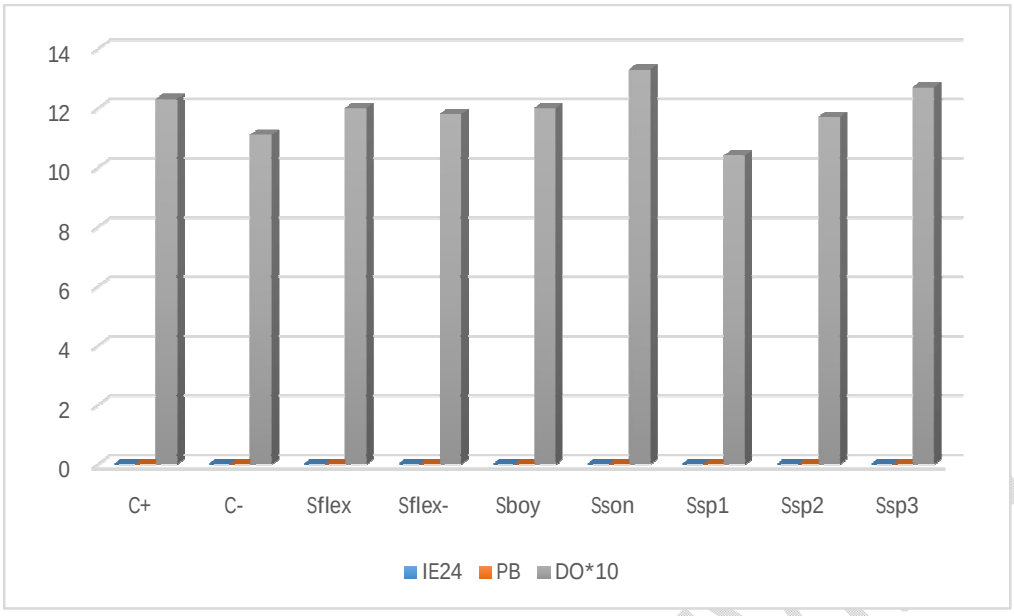
(b)

Figure 7 : 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.

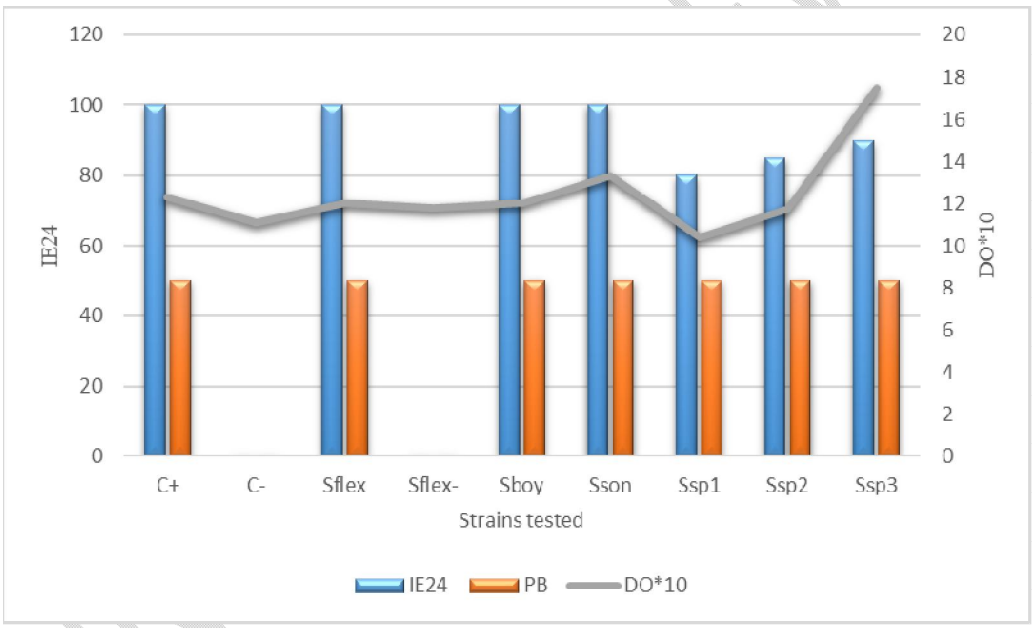
Legend: C+: *S. flexneri* 5a M90T grown aerobically; C-: negative control (*S. flexneri* 5a M90T *spa* 40-); Sflex: *S. flexneri* 5a M90T, Sflex: *S. flexneri* 5a M90T *spa* 40-; Sson: *S. sonnei*; Sboy: *S. boydii*; Ssp1-3: hospital *Shigella* sp.

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 (Figure 8).



(a)



(b)

Figure 8 : 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.

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 (Figure 9). 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 (**Figure 9**).

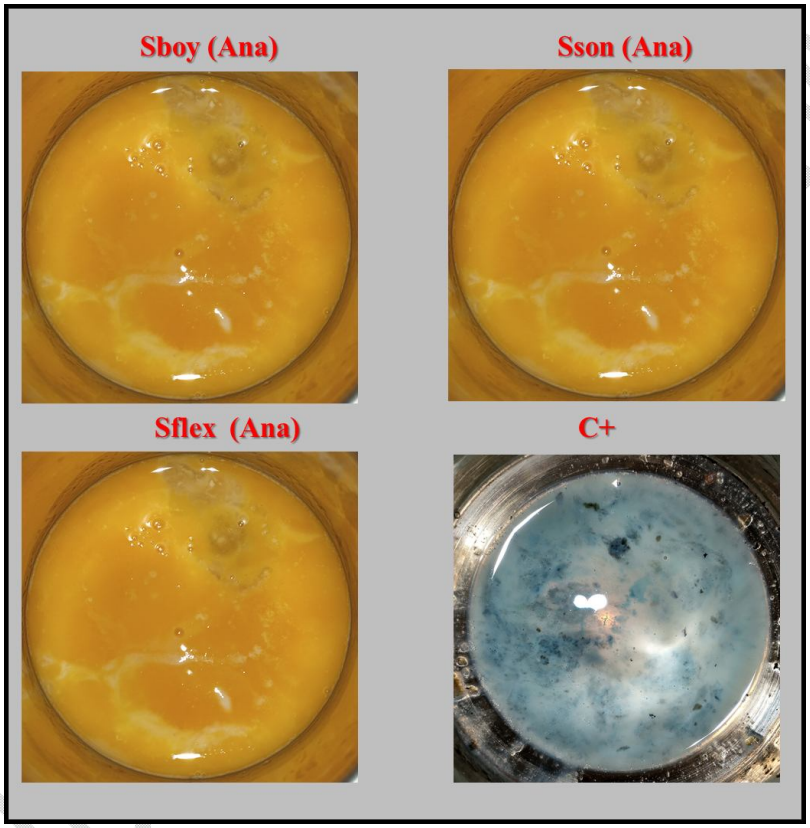


Figure 9 : Appearance of the egg's contents following the test for anaerobic contamination.

Legend: C+: *S. flexneri* 5a M90T grown aerobically; Sflex: *S. flexneri* 5a M90T; Sson: *S. sonnei*; Sboy: *S. boydii*; Ssp1-3: hospital *Shigella* sp.

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 I below.

Table I : *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

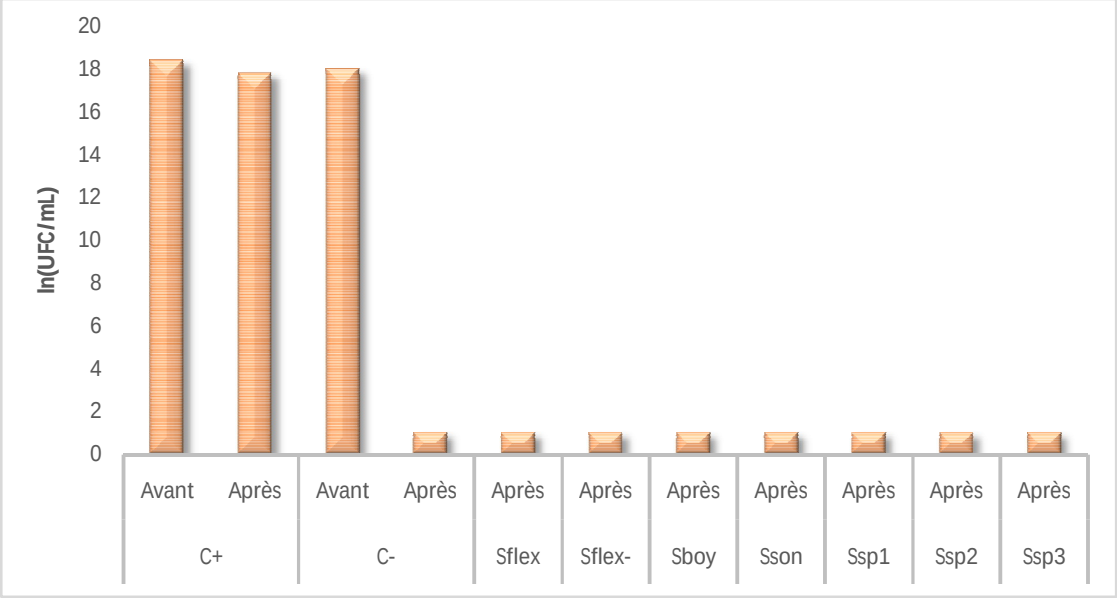


Figure 10 : *Shigella* spp. strains' invasiveness in anaerobic environments.

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..

Discussion

This research was conducted to advance knowledge of the mechanism by which *Shigella* spp. invade epithelial cells. It aims to describe specifically how *Shigella*-genus bacteria behave when producing and using biosurfactants as a virulence factor in the colonization of epithelial cells under anaerobic conditions.

Ninety-three (93) different *Shigella* spp. strains, including four (04) reference strains found through molecular biology methods (sequencing), and three (3) strains found through genus-based identification (biochemical reaction and culture characters on boxes), were used in this study.

We established that the strains used in this study belonged to the genus *Shigella* by combining the characteristics of plate culture, the shape of the bacterial cells after GRAM staining, the mobility of the cells after fresh observation, and the degradation of lactose in CLED medium. *Shigella* bacteria are, in fact, Gram-negative, immobile, do not ferment or ferment, lactose slowly, and the colonies on SS and Hektoen medium are colorless. The gene for kanamycin resistance is present in the genome of the Spa40- mutant.**[12]**. For this purpose, Kanamycin is a selection antibiotic to confirm the isolation of *S. flexneri* 5a M90T spa40- **[12]**. The *S. flexneri* 5a M90T wild-type strain demonstrates a comparable level of streptomycin resistance. These antibiotics were used to choose and phenotypically confirm the purified *Shigella* strains that would be used in the rest of this study **[9]**. All strains thought to be members of the *Shigella* spp. group had restriction fragment profiles that matched those of the reference strains that had previously been molecularly identified. This result provides unambiguous proof that the strains isolated from the sampling sites belonged to the genus *Shigella*.

Shigella spp. strains used in this study have all demonstrated the capacity to grow both with and without oxygen. **[13]**. The appearance of colony-forming units on the agar's surface after 24 hours, which is a sign of bacterial growth, serves as a measurement of this growth. The multiplication or expansion of the bacterial biomass within the medium is referred to as bacterial growth. Growth in liquid media is measured macroscopically by the appearance of cloudy culture medium and microscopically by optical density. This outcome supports *Shigella's* facultative anaerobic phenotype, which is a type of bacteria. *Shigella*, members of the Enterobacteriaceae family, 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. **[14]**.

The emulsification test is an indirect test that demonstrates the production of substances with surface active properties in the extracellular medium that are capable of lowering the surface tension between two different phases (water/oil air/water, hydrophobic/hydrophilic), or between two membrane interfaces. The ability of specific bacteria to secrete biosurfactants can be highlighted through the emulsification test.**[15].** Due to their capacity to form micelles, these molecules enable the coexistence of two potentially incompatible molecules in the same phase. *Shigella* able to produce and secrete biosurfactants when there was oxygen leakage and SST3 was being induced, according to Kinavouidi and colleagues. The same study contends that these biomolecules may be crucial to the pathogenesis of *Shigella***[9]**. The attachment of

bacteria to plant cells is facilitated by biosurfactants, which are found in bacteria like *Shigella* and *SalmonellaenteridisTyphimurium* SE 86, which belong to the same family **[16]**and thus participates in their pathogenesis, by promoting the persistence of the germ in the environment and by coordinating their resistance to antiseptics.

Using cell-free supernatants from anaerobic cultures of *Shigella* spp. strains for a 24-hour period, an emulsification test revealed that all strains—aside from the spa40 mutant—were capable of emulsifying gasoline and diesel with emulsification index values ranging from 60 to 100%. These findings support those made by Kinavouidi and coworkers in 2020**[9]** and Cynthia Alias-Villegas and staff in 2022 **[17].** Biosurfactants are compounds that have the ability to emulsify hydrocarbons **[15].** The positive control wild strain grown without oxygen and the same strain grown aerobically had comparable index values. Whether the medium was oxic or anoxic, *Shigella* spp. maintained its emulsifying ability. Contrary to the aforementioned studies, which were carried out in an oxygen-rich environment, this is the first study to show that *Shigella* spp. can emulsify under conditions that mimic invasion conditions, such as colonization and dissemination within the colonic epithelium.

Biosurfactants have been implicated in a number of multicellular phenomena in *P. aeruginosa*, including the formation of biofilms and swarming movements on semi-solid media **[15, 18]**. Besides, *Pseudomonas aeruginosa*, swarming has been described to *Proteus mirabilis***[18]**, *Salmonella* sp. **[19]**, and *Shigella***[20]**. This study demonstrated that all *Shigella* spp. strains tested could swarm on semisolid media under anaerobic conditions, with swarm diameters between 75 and 80 mm, except for the *S. flexneri* 5a M90T spa40-mutant. The positive control, which was examined under oxygen-rich conditions, also produced a pattern that was similar. This finding demonstrates that *Shigella* can swarm both 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 between a strain's capacity to produce biosurfactants and its propensity to swarm has been found in *P. aeruginosa.*

If any biosurfactant is secreted into the extracellular medium into the culture medium, it lowers the tension between the agar and the bacterial outer membrane and encourages *Shigella* bacterial cells to slide or slide on the agar surface, leading to the swarming phenotype. Additionally, it was established that the bacterial flagellum is not necessary for the swarming phenotype. This study supports the findings of the earlier investigation conducted in 2020 by Kinavouidi and colleagues **[9]**. 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, and it would give *Shigella* spp. a cytolytic power in addition to the effectors typically described for SST3. It is possible that the biosurfactant will disrupt the colonic epithelium and impair mucosal immunity-related cell function.

This study demonstrated *Shigella* spp.'s capacity to form biofilms in a similar manner. In fact, biofilm development is a reaction to extreme cellular stress. The presence of a high concentration of bile salts in the digestive tract and gastric acidity are stress factors that affect *Shigella* in its pathogenesis under normal circumstances. This study thus supports the finding that Shigella spp. can form biofilms without oxygen.

These bacterial species' biofilms are created in part by P. aeruginosa rhamnolipids. In light of the findings of this study, we think the *Shigella* biosurfactant would perform the same function. In fact, not every factor causing *Shigella* spp. to form biofilms has been distinctly identified. *Shigella* spp.'s capacity to produce and secrete biosurfactants hasn't been previously studied, so a cause-and-effect connection couldn't be proven. However, this study demonstrated that the quantity 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. Accordingly, there is a correlation between these three constants: the optical density that characterizes the amount of bacterial biomass present, the ability of the strains to secrete biosurfactants, and the ability of the strains to form biofilms.

The *S. flexneri* 5a M90T *spa40*-strain was unable to produce biofilms or emulsify hydrocarbons. This finding suggests that the two occurrences are in fact related. The biomolecule responsible for emulsification and biofilm formation would be secreted via SST3 and would be an effector involved in the virulence of *Shigella* spp. Kinavouidi and colleagues in 2022 found that this strain has a defective type 3 secretion apparatus **[20]** showed that the production of biosurfactants was first regulated by SST3 in the early stages of infection and then by quorum sensing. This study confirms that *Shigella* spp. in its pathogenesis uses two regulatory systems: the first one completely dependent on SST3 itself, and the second one mediated by quorum sensing. Quorum sensing is a bacterial concentration-dependent gene regulation system.

The ability of *Shigella* to form biofilms was described by Kourtney and colleagues **[21]**. 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 invasion-mimicking chicken egg contamination test, showing that they are unable to enter embryonic yolk cells. In fact, none of the tested strains showed any growth on the culture medium after 24 hours of anaerobic conditions at 37 °C. The results of the emulsification test conducted on the egg contents 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 positive control that was carried out aerobically, or with oxygen present, was successful and permitted the isolation of colony-forming units on particular media after 24 hours. This finding emphasizes the lack of cell invasion by *Shigella* spp. strains and the crucial part 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 SST3, so the fact that none of the *Shigella* strains in this study invaded is due to SST3's ability to function. SST3 is oxygen-dependent, as demonstrated by recent research by Benoît Marteyn and colleagues. Without oxygen, SST3 is inhibited by a factor called FNR (fumarate-nitrate reductase), which in turn inhibits Spa33 and Spa32 **[13]**. Spa33 is one of the subunits of the Spa47 ATPase responsible for the production of the proton motive force that provides the energy for the translocation of effectors from the bacterial cytosol to the cytoplasm of the host cell **[13]**.

Spa32 is an SST3 molecule that acts as a molecular ruler and determines the size of the needle, a sine-qua-non condition for good SST3 activity. Indeed, Kayath and colleagues in 2008 demonstrated that *Shigella* with a longer or shorter than normal size was unable to invade **[22]**. We also think that Kayath and colleagues' findings may help to explain why the tested bacterial cells were unable to invade or contaminate the eggs, as a result of Spa33 and Spa32 being simultaneously inactive and repressed by the FNR factor (SST3 regulator). Our findings are consistent with those of Benoit Marteyn and associates, who also showed that *Shigella* spp. could not colonize epithelial cells **[13]**.

The nuanced findings regarding the strains' capacity to invade cells in the presence or absence of oxygen supports the conclusions made by Kinavouidi and colleagues regarding the pathways by which *Shigella* secretes biosurfactants. As shown by Kinavouidi and associates **[20]**, *Shigella* has developed other mechanisms of adaptability 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 via SST3, the biosurfactant must be either totally or partially

proteinaceous as suggested by Kinavouidi and colleagues **[9]**. SST3 is the only pathway involved in biosurfactant secretion in *Shigella*, unlike *P. aeruginosa*.

Inhibition of Spa33 and Spa32 in *Shigella* spp. helps to explain the leakage of SST3 because as described by Claude Parsot in 2009 **[14]**, SST3 is simultaneously activated by contact with the host epithelial cell. Thanks to the villi rich in blood vessels, the residual oxygen diffusing from the capillaries allows the inhibition of SST3 to be lifted by diffusion of oxygen inside the bacterial cell**[13]**. Indeed, in the absence of oxygen and consequently in the absence of cell contact, SST3 is partially assembled and consequently cannot allow the escape of certain effectors, among them the biosurfactant. This result is indeed correlated with the phenotype of the Spa40-mutant.

Conclusion

The present work has shown that: (i) *Shigella* bacteria are able to produce and secrete biosurfactants in the extracellular medium under anaerobic conditions; (ii) *Shigella* bacteria are able to swarm on semisolid media under anaerobic conditions; (iii) *Shigella* bacteria are able to form biofilms under anaerobic conditions; (iv) biosurfactants produced by *Shigella* are considered involved in biofilm 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 SST3 conditions.

Data Availability

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

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