Advances in Microbiology, 2022, 12, 270-294
https://www.scirp.org/journal/aim
ISSN Online: 2165-3410
ISSN Print: 2165-3402

Shigella Strain Has Developed Non-Studied Pathogenicity Mechanisms of Adaptability in the Colonization of Epithelial Cells

Duchel Jeanedvi Kinouani Kinavouidi[1,2], Christian Aimé Kayath[1,2*], Nicole Prisca Makaya Dangui Nieko[1,3], Saturnin Nicaise Mokemiabeka[1], David Charles Roland Moukala[1], Etienne Nguimbi[2]

[1]Laboratoire de Biologie Cellulaire et Moléculaire (BCM), Faculté des Sciences et Techniques, Université Marien Ngouabi, Brazzaville, République du Congo
[2]Institut National de Recherche en Sciences Exactes et Naturelles (IRSEN), Brazzaville, République du Congo
[3]Ecole Normal Supérieur (ENS), Université Marien Ngouabi, Brazzaville, République du Congo
Email: *chriskayath@yahoo.fr

How to cite this paper: Kinavouidi, D.J.K., Kayath, C.A., Nieko, N.P.M.D., Mokemiabeka, S.N., Moukala, D.C.R. and Nguimbi, E. (2022) *Shigella* Strain Has Developed Non-Studied Pathogenicity Mechanisms of Adaptability in the Colonization of Epithelial Cells. *Advances in Microbiology*, **12**, 270-294.
https://doi.org/10.4236/aim.2022.124020

Received: January 22, 2022
Accepted: April 25, 2022
Published: April 28, 2022

Abstract

According to the World Health Organization, foodborne diseases are a major public health problem, particularly in developing countries including the Republic of Congo. They are responsible for several episodes of diarrhea, especially in children under five years old. There is no reliable epidemiological data on the pathogenicity of the *Shigella* spp. strains circulating in the whole Republic of Congo drafting this paper. The purpose of this study was to examine the *Shigella* spp strain pathogenicity close to an environment contaminated with faeces in the city of Brazzaville. As a result, 54 isolates have been associated with *Shigella* spp. The gastric acid resistance test performed on *Shigella* Environmental Strain (SES) and *Shigella* Clinical Strains (SCS) resulted in 38.8% (21/54) and 100% acid resistant, respectively. *Shigella* spp. Strains (SES and SCS) were ranged in a survival percentage from 11% to 93%. By monitoring Biosurfactant-Like Molecule (BLM) production, we showed that the BLM production of SES and SCS was highly dependent on bacterial culture density involving the Quorum Sensing (QS). *S. flexneri*, *S. boydii*, and *S. sonnei* and as well as SES and SCS were able to invade and contaminate eggs by colonizing egg yolk. The counting bacteria were ranging from two to 5 $\times 10^7$ CFU/mL after contamination. Concomitantly, BLM was secreted during the post contamination of poultry eggs with 100% EI24. Further by trying to show the pathogenicity by the hemolysis test, we have shown that SES and SCS were able to show significant areas of lysis on blood agar. Finally, this work has proposed an additional model of cell invasion including biosurfac-

tants during the pre- and post-invasion phases.

Keywords

Shigella, Biosurfactant, Quorum Sensing, Invasion, Epithelial Cells, Pathogenicity

1. Introduction

Shigella is highly pathogenic bacteria specific to humans and some primates. This group of bacteria belongs to the Enterobacteriaceae family, they are aero-anaerobic, facultative, immobile, non-encapsulated, sporeless, Gram-negative with a size of 2 to 3 μm long by 0.5 to 0.7 μm wide [1]. Indeed, 10 to 180 bacteria are enough to promote the disease steps [2]. In sub-Saharan Africa and Asia, this disease is responsible for 54,900 deaths per year, and nearly 164,300 worldwide. Children under 5 years of age are the most vulnerable group to the disease [3]. Shigellosis is a disease of poor hygiene and is carried by flies that contaminate food and drink intended for human consumption, like enteropathogenic. *Escherichia coli* and *Brucella* spp. *Shigella* is also able to withstand gastric acidity (pH 1 - 3), which is characterized by invasion and inflammatory destruction of the human colonic epithelium [4] [5]. The ability of *Shigella* spp to colonize the colon is aided by a type 3 secretion apparatus (T3SA), which acts as an injectisome, allowing translocation of virulence proteins from the bacterial cytosol to the cytoplasm of the host cell resulting in the invasion of the colonic epithelium by *Shigella* spp [6] [7]. The genetic basis for *Shigella* invasion of epithelial cells is a 31 kb region carried by the virulence plasmid pWR100 called the "entry region" which encodes two operons: the *ipa-ipg* operon which encodes *Shigella* effector proteins and their chaperones; the mxi-spa operon which encodes structural proteins of the T3SA [7].

A previous study conducted by our laboratory team showed that *Shigella sonnei*, *S. boydii*, and *S. flexneri* had the ability to produce and secrete biosurfactants in the extracellular environment. This study postulated that these biosurfactants are involved in the invasion of epithelial cells by *Shigella*. *Pseudomonas aeruginosa*, *Proteus mirabilis*, and *Bacillus subtilis* are also able to carry out multicellular phenomena such as swarming by using the production of biosurfactants in the extracellular medium [8].

Several studies have reported that biosurfactant production, especially in *P. aeruginosa* or *B. subtilis*, is regulated by Quorum sensing [9] [10]. Moreover, biosurfactants promote multicellular phenomena such as biofilm formation and swarming motility. These observations have been made in many bacterial species except for *Shigella* [11] [12]. From an epidemiological point of view, in the Republic of Congo, there are no data on the pathogenicity of the circulating strains of *Shigella* spp., either in hospitals or in the environment. As a faecal peril disease, people living in the outskirts of Brazzaville and the Pool South region use

wells and boreholes as a source of water. These practices are increasingly exposed to Shigellosis. Thus, the present study aims to evaluate the pathogenicity of *Shigella* spp strains isolated from wastewater and faecally contaminated soils in the outskirts of Brazzaville.

2. Methods

Strains and Culture Conditions

Four (4) *Shigella* laboratory strains (*S. flexneri*, *S. flexneri* Spa40-, *S. sonnei*, and *S. boydii*) were used as reference strains, four (4) *E. coli* strains transformed with pGEX4T1 and its derivatives (pGEX4T1: ipaB, C, D), provided by the Laboratory of Molecular Bacteriology at the Université Libre de Bruxelles (ULB), three (3) pure culture strains were isolated from patients admitted to Hospital University Center of Brazzaville to 2018 (provided by the Bacteriology Laboratory). A strain of *P. aeruginosa* has been used as a control [4]. By using wastewater and soil contaminated with faeces, dilutions were done, and the bacterial suspension was streaked on SS and Mac Conkey media as described by the manufacturers. 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.

Study of the Pathogenicity of *Shigella* Strains Isolated from the Environment

S. flexneri, *S. flexneri* Spa40-, *S. sonnei*, *S. boydii*, *Shigella* Environmental Strain (SES), and *Shigella* Clinical Strains (SCS) have been spread on Petri dishes containing LB medium added to Congo red and containing the appropriate antibiotics for 24 hours at 37˚C. Then the colonies have been inoculated in 10 ml of LB + antibiotic broth for 24 hours at 37˚C. From 24 h culture, 1 mL of the culture was inoculated in 50 mL of LB broth with a suitable antibiotic at 37˚C with shaking (250 rpm) for 24 hours until reaching an OD between 0.6 and 0.8 at 600 nm. 1 ml of this culture was stock and the rest was centrifuged at 13,000 rpm. The pellet was separated from the supernatant and stored at −20˚C.

Acidic pH Resistance Test

The overnight culture was diluted at 1:100 in LB medium acidified with HCl, *i.e.*, 10 µL of the overnight culture in 990 µL of LB at pH 2. The latter is incubated at 37˚C for 2 hours. A series of dilutions (up to 10^{-3}) were then made from the 2 H culture. A volume of 100 µL of each dilution was inoculated onto SS and then incubated at 37˚C for 15 h. A count was performed after incubation to assess the viability of the strains at this pH.

Calculation of the Survival Rate of Bacterial Cells Grown at Acidic pH

The survival rate of bacterial cells is defined as the percentage of viable cells after acid stress compared to the initial number of viable cells (neutral pH) in the initial state.

$$\%\ \text{survie} = \frac{\text{Number of viable cells at pH2}}{\text{Number of viable cells at neutral pH}} \times 100$$

Emulsification Test (E24)

The emulsion index (E24 or E48) was calculated as an indicator for biosurfactants production. The medium was adjusted to pH 7.2 and supplemented with gasoline or diesel fuel (1 mL for 300 mL of medium). This experiment was done in triplicate. The E24 or E48 was investigated by adding crude oil to LB medium in a 1:1 ratio (v/v). The solution was vortexed for 5 min and incubated for 24 h. The emulsion rate was calculated through the height of the emulsion layer. In addition, E24 was determined for gasoline and diesel fuel hydrocarbons. All experiments were performed in triplicate.

$$\text{E24} = \text{height of emulsion layer} / \text{total height of solution} \times 100$$

Shigella strains with positive emulsification have been assessed by using an acid precipitation test. Briefly, overnight culture (OD about 0.6) was centrifuged at 13,000 ×g for 15 minutes. Once the supernatant was collected, it was acidified by adding concentrated HCl to a pH of 2.0 using an instillator. We allowed the precipitate to form at 4˚C overnight. We then centrifuged the night mixture at 13,000 g for 15 minutes to obtain granules. The granules obtained were tested (emulsification test) to evaluate their ability to emulsify the hydrocarbons.

Bacterial Swarming Assays

Swarming was studied for all *Shigella* strains and *P. aeruginosa* used in this study. Using plate assays containing 0.5% noble agar and LB medium with 0.5% dextrose. The mixture was heated at 121˚C, for 15 min. After sterilization, the medium was supplemented with adequate antibiotics including streptomycin 100 µg/mL and kanamycin 50 µg/mL for all *Shigella* strains. Approximately 6 h after pouring the plates, they were inoculated using a sterilized platinum wire with log-phase cells (optical density at 600 nm [OD600] 0.6) grown in their respective media used for the swarm experiments. Swarming plates that were imaged only for their comparative endpoint swarming development (*i.e.*, analysis of different strains) were incubated at 30˚C for 48 h prior to imaging [13].

Induction Test by Congo Red Reagent

From the 24 h culture, 1 mL of the culture has been inoculated into 49 mL of LB broth with a suitable antibiotic at 37˚C under agitation (250 rpm) for 24 h until an OD between 0.6 and 0.8 was reached at 600 nm. 1 mL of this culture was taken as stock and the rest was centrifuged at 13,000 rpm. To the pellet obtained after centrifugation, we added 500 µl of sterile PBS and 10 µl of Congo Red (10 mg/mL) and then rapidly mixed in PBS without shaking the solution to avoid damaging the "secretion". The sample has been carefully put in an Eppendorf tube and incubated at 37˚C under agitation. After 30 minutes, we centrifuged the solution at 15,000 rpm for 15 minutes. We carefully removed the supernatant which we used for the emulsification test.

Correlation of Quorum Sensing Activity and Biosurfactant Production

To standardize the concentrations of salicylic acid and D-galactose during the growth of the bacteria, viability testing of *Shigella* strains with salicylic acid and D-galactose had been done first all. The *S. flexneri* 5a M90T strain was grown in LB medium in the presence of a D-galactose concentration gradient ranging from 10 µM to 100 mM and a Salicylic acid gradient ranging from 0.25 to 50 mg/mL. The strains were cultured under agitation (250 rpm) at 37˚C and the evolution of the optical density over time was monitored for 48 h. 20 µL of the cultures with normal growth was spread out on plates and incubated at 37˚C for control.

Effect of D-Galactose and Salicylic Acid on Biosurfactant Production

From the overnight cultures of the *Shigella* strains tested, 1 mL of the culture was inoculated into 49 mL of LB broth (with the appropriate concentrations of D-galactose and Salicylic Acid) at 37˚C under agitation (250 rpm) for 24 hours until an OD of 0.6 to 0.8 was reached at 600 nm. The pellet was separated from the supernatant by centrifugation at 15,000 rpm and then, using an emulsification test, we evaluated the emulsifying index of the hydrocarbons. In addition, 20 µL of the culture have used to perform the swarming test to evaluate the ability of *Shigella* strains to move on a semisolid medium.

Egg Contamination Test or Bacterial Invasion Test

A 1 mL volume of an overnight bacterial culture (OD between 0.6 and 0.7) was transferred to 49 mL of LB containing a poultry egg. The culture medium was supplemented with selection antibiotics streptomycin 100 µg/mL for wild type and strains. kanamycin 50 µg/mL for the mutant spa40-. This culture was shaken (140 rpm) at 37˚C for 72 hours. The contaminated eggs were previously washed with ethanol 100%.

To characterize bacterial invasion, the eggs were assessed by the deterioration of the shell, the morphology and the physicochemical quality of the eggs and the counting of bacteria after 72 hours of contamination have been done.

Briefly**,** after 48 - 72 h of culture, eggs were aseptically removed from the culture medium and broken up using a heated platinum seeder. The egg contents were poured into a glass vial (previously sterilized at 121˚C for 2 H) and homogenized with a vortex. 1 mL of each contaminated sample was transferred separately to a test tube containing 9 mL of sterile physiological water. After 1 min of homogenization with a vortex mixer (VELP Scientifica, Advanced vortex mixer, Italy), decimal dilutions were made from the stock solution of each sample. The different dilutions (10^{-1} to 10^{-5}) constitute the inoculum from which we performed the bacterial enumeration on the SS medium with the adequate antibiotic, kanamycin 50 µg/mL, and Streptomycin 100 µg/mL as the final concentration. The production of the biosurfactant-like molecule (BLM) has been tested as previously described.

Performing the Interaction between IpaB, C, and D by Using GST Pull-down Assay

Plasmids pGEX4T1, expressing the fusion protein GST-IpaB, C, and D, were

kindly provided by the molecular bacteriology lab of the Free University of Brussel [7].

E. coli strain Top10, harbouring pGEX4T1 or its derivatives and expressing GST-IpaB, C, or D, was cultured in LB broth with ampicillin 100 µg/ml for 2 h at 37˚C, then IPTG was added to a final concentration of 0.1 mM. After incubation for 3 h at 30˚C, bacteria were harvested and GST fusion proteins were purified as described by the manufacturer by using Glutathion Sepharose 4B (Amersham Pharmacia Biotech).

To highlight and assess the protein-biosurfactant interaction, the matrix was mixed with the biosurfactant extract and then washed with PBS four times). Elution has been done with 50 µL of Glutathione. For the eluates, we performed emulsification tests to demonstrate the interaction between the proteins of the translocon, the Tip T3SS, and the biosurfactants produced.

Statistical Analysis

The data represent the arithmetical averages of at least three replicates. Data were expressed as mean ± SD and Student's t-test was used to determine statistical differences between strains and $p < 0.05$ was considered significant. GraphPad Prism 7 software; Excel 2013 software was also used for the analysis and graphing of results.

3. Results

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

A total of 63 strains were used in this study. 54 strains of *Shigella* spp were isolated from soil and wastewater from the outskirts of *Brazzaville* South. These strains were isolated from Mac Conkey medium and purified on SS medium. Four (4) reference strains of *Shigella*, one reference strain of *P. aeruginosa*, and one hospital strain of *Salmonella* spp were used as controls. The *E. coli* Top 10 strains used in this study were constructed at the Laboratory of Molecular Bacteriology of the Free University Brussels. 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. Strains were able to grow in the presence of streptomycin.

Study of the Pathogenicity of *Shigella* spp. Strains Isolated from the Environment

The acid resistance test was used to select potentially pathogenic strains of *Shigella* spp. Among fifty-four (54) strains of *Shigella* spp purified from SS with streptomycin selection medium, twenty-one (21) strains were resistant to acidic pH as shown in **Figure 1**. The percentage of survival was between 10% and 95%. The wild-type strain of *Shigella flexaneri* M90T, *Shigella boydii*, *Shigella sonnei*, *Salmonella* spp. as well as the clinical strains of *Shigella* (SC1, 2, and 3) had the highest percentages. Environmental strains of *Shigella* including SE4 and SE10 showed very high percentages of acid resistance. Some of them were about 40 and 60% of survival (**Figure 1**).

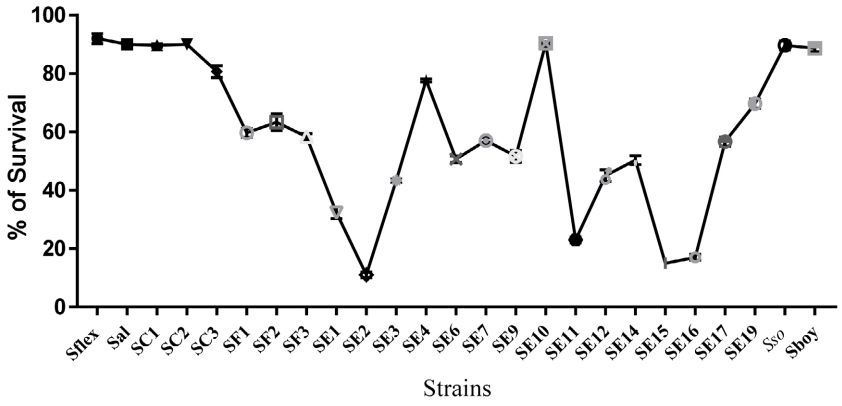


Figure 1. Acid resistance profile of environmental and clinical strains of *Shigella* spp. used in this study. S.flex: *S. flexneri* M90T; Sso: *S. sonnei*; Sboy: *S. boydii*; Sal: *Salmonella* spp.; SC: clinical strains; SF: environmental strain isolated from soil contaminated with faeces; S.E: environmental strains of Shigella spp. isolated from sewage.

Screening for Biosurfactant Production

Strains that were resistant to bacterial acidity were randomly selected to evaluate their ability to emulsify hydrocarbons. All strains tested emulsified hydrocarbons with EI24 ranging from 33% to 100% after 24 hours (**Figure 2(a)**). *P. aeruginosa*, all reference strains of *Shigella* and *Salmonella* spp with emulsification index of 100%. The *S. flexneri* 5a M90T Spa40-strain was used as negative control (**Figure 2(b)**).

The Ability of *Shigella* spp. Strains to Swarm

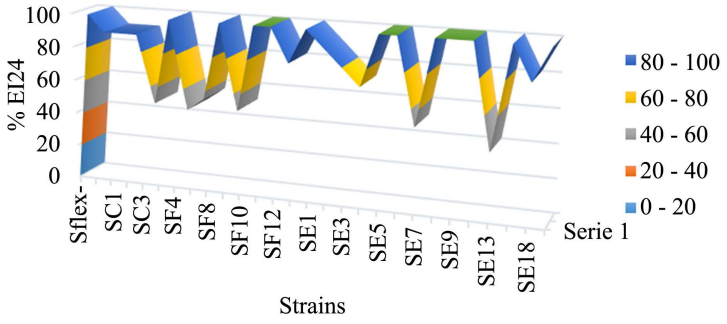
Swarming is considered a virulence factor mediated by the production and secretion of biosurfactants into the extracellular environment. To demonstrate the ability of *Shigella* strains isolated from the environment to swarm, we performed the swarming test. This study showed that all *Shigella* spp were able to swarm on semisolid media as shown in **Figure 3**.

The Correlation between Emulsifying Capacity and Bacterial Density

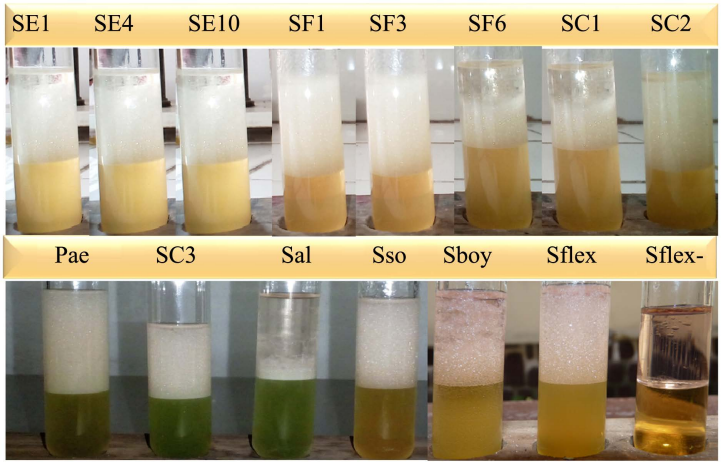
The regulatory pathways of biosurfactant production in *Shigella* spp have not yet been demonstrated. In this study, we monitored the evolution of emulsifying capacity as a function of growth using the reference strain *S. flexneri* 5a M90T. The growth was evaluated as a function of the optical density at 600 nm. Our study showed that *S. flexneri* 5a M90T is unable to emulsify hydrocarbons with the OD down to 0.6. However, from the exponential growth phase onwards, biosurfactants are produced and secreted into the extracellular medium with an optical density (OD) up to 0.6 (**Figure 4**).

Quorum Sensing Inhibition Assay

Salicylic acid and D-galactose are molecules that can interfere with bacterial growth at high concentrations. We examined the growth behaviour of *S. flexneri* 5a M90T in the presence of a range of concentrations of D-galactose and salicylic acid. Our study showed that *Shigella* could normally grow in LB medium at concentrations between 10 µM - 100 mM D-galactose (**Figure 5(a)**) and 1.25 mg/mL salicylic acid (**Figure 5(b)**).



(a)



(b)

Figure 2. Emulsification test of some clinical and environmental strains of *Shigella* spp. isolated in Brazzaville. (a): Range of hydrocarbon emulsification by Shigella bacteria used in this study; (b): photo showing the result of the test for emulsification of hydrocarbons by bacteria of the genus Shigella of clinical and environmental origin. Here we have shown some of them for illustrative purposes. S.flex: *S. flexneri* M90T; SC: clinical strains; SF: environmental strains isolated from soil contaminated with faeces; S.E: environmental strains of *Shigella* spp. isolated from sewage. Colour code: blue (0% - 20%), brown (20% - 40%), grey (40% - 60%), yellow (60% - 80%), and light blue (80% - 100%).

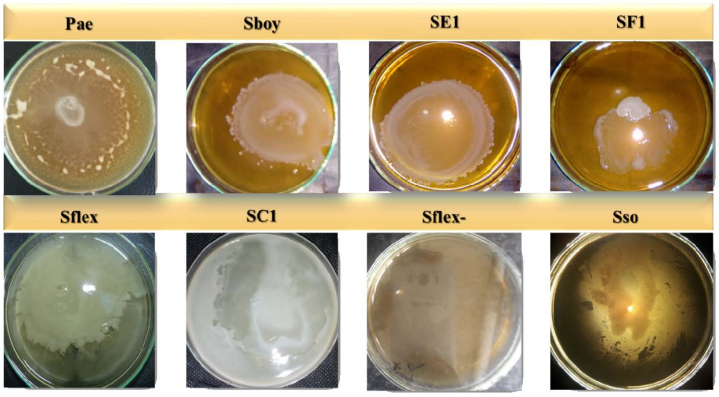


Figure 3. Ability of *Shigella* spp. strains to swarm on semisolid medium or swarming profile on soft agar. S. flex: *S. flexneri* M90T used as a positive control; SC: clinical strain of *Shigella* spp.; SE: environmental strain of *Shigella* sp isolated from sewage; SF: strains of *Shigella* spp. isolated from faecally contaminated soil.

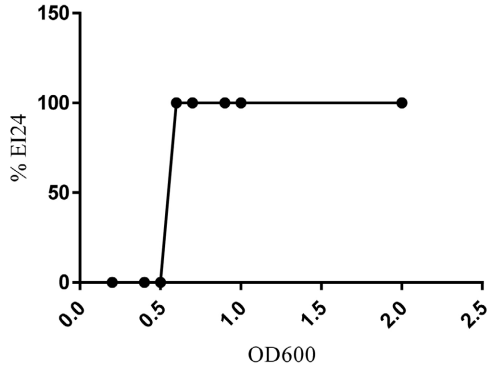
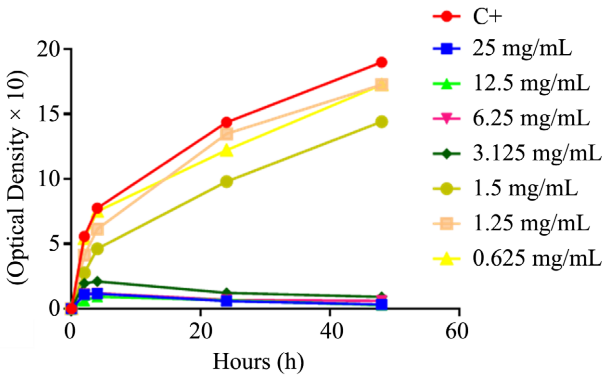
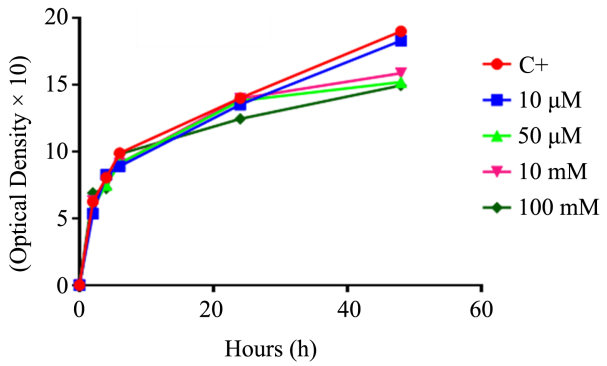


Figure 4. Monitoring the emulsifying capacity of *S. flexneri* M90T as a function of optical density. % IE24: Emulsification index; OD: optical density.



(a)



(b)

Figure 5. Viability of *S. flexneri* 5a M90T strains in the presence of different concentrations of inhibitors. (a): this figure shows the ability of *Shigella* strains to tolerate or not D-Galactose at different concentrations; (b): this figure shows the ability of *Shigella* strains to tolerate or not D-Galactose at different Salicylic Acid concentrations. C+ represents the *Shigella flexneri* M90T strain tested in the absence of inhibitors (D-galactose and Salicylic acid).

In terms of D-galactose, all concentrations used in this study were not lethal for the bacteria. On the other hand, an interesting growth was observed at 10 µM as the growth curve merged with that of the control (Figure 5(a)). In Figure 5(b), bacteria were not able to grow between 2.5 and 3.25 mg/mL. At these concentra-

tions, the bacteria are not viable. On the other hand, from 1.5 mg/mL the bacteria are viable. However, optimal growth is observed at 1.25 mg/mL. The curve is close to the positive control which was grown without an inhibitor.

Effect of Salicylic Acid and D-Galactose on the Ability to Produce Biosurfactants

The ability of the strains to emulsify hydrocarbons in the presence of salicylic acid and D-galactose was evaluated. Our study showed that at concentrations of 1.25 to 0.5 mg/mL salicylic acid, all *Shigella* strains tested lost their emulsifying ability (**Figure 6(a)**). However, at 0.25 mg/mL salicylic of acid, these strains recovered their ability to emulsify hydrocarbons (**Figure 6(a)**). This study also showed that at D-galactose concentrations between 10 µM and 100 mM, the emulsifying capacity of hydrocarbons by bacteria of the genus *Shigella* was inhibited (**Figure 6(b)**). Emulsification profiles of *Shigella* sp strains used in this study are illustrated in **Figure 6(c)** and **Figure 6(d)**).

Effect of Salicylic Acid and D-Galactose on the Ability of Strains to Swarm on Semisolid Media

We did not find any studies in the scientific papers showing the effect of these molecules on multicellular phenomena such as swarming. Our work showed that at concentrations of 1.25 mg/mL for salicylic acid and 10 µM of D-galactose, all strains were unable to swarm on a semisolid surface. However, in the absence of these molecules, the ability to swarm on a semisolid medium was restored (**Figure 7**).

The Ability of Shigella Strains to Invade Epithelial Cells

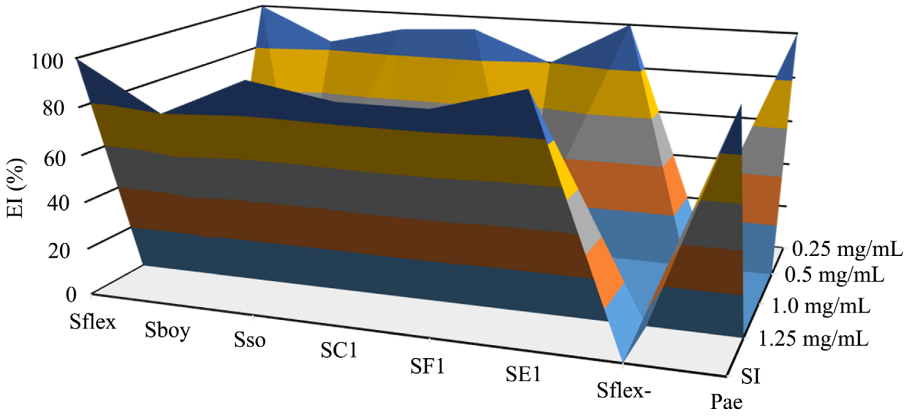
Hemolysis Assay of Clinical and Environmental *Shigella*

To highlight the ability of *Shigella* sp strains isolated from the environment to interact with biological membranes, we wanted to observe the behaviour of these bacteria on a Poly (Blood Agar) medium. This work showed that except for *S. flexneri* 5a M90T spa40-, all strains showed beta haemolytic activity resulting in the appearance of a clear halo of lysis around the colonies as illustrated in **Figure 8** below.

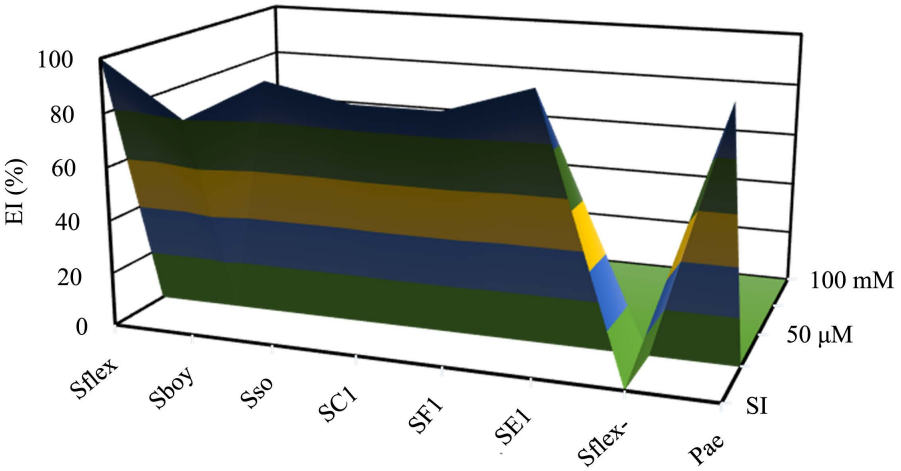
Egg Contamination Test with Shigella spp. Isolated from the Environment

To assess the ability of environmentally isolated *Shigella* sp strains to invade cells, the egg penetration or contamination test was performed. After 72 hours of incubation at 37˚C with agitation, the eggs submitted to this study showed macroscopic rotting. The rotting was assessed by physicochemical changes in the eggs compared to the fresh control egg, including a putrefying odour indicative of rotting, abnormal coagulation of the yolk which had turned black, and fluidification of the albumen or egg white. The contaminated eggs were revealed microscopically after culture on SS + streptomycin medium, the appearance of typical *Shigella* sp colonies at variable densities after 72 h incubation at 37˚C as illustrated in **Figure 9(a)**.

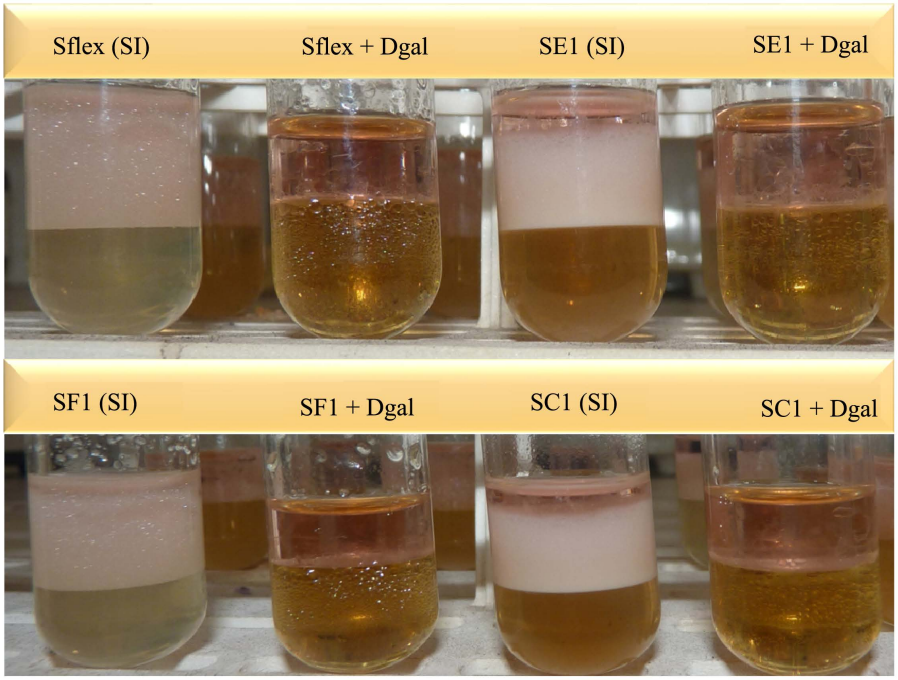
As an internal control, colonies with the cultural characteristics of streptomycin-resistant *Shigella* spp. were characterized by performing the GRAM test, the


100
80
60
40
20
0
EI (%)
Sflex
Sboy
Sso
SC1
SF1
SE1
Sflex-
Pae
SI
0.25 mg/mL
0.5 mg/mL
1.0 mg/mL
1.25 mg/mL

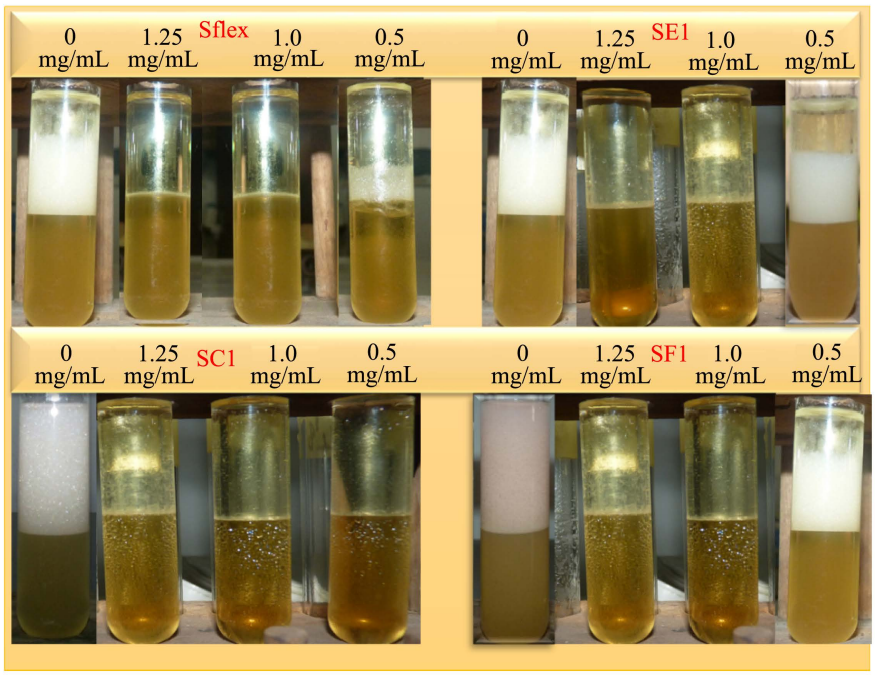
(a)


100
80
60
40
20
0
EI (%)
Sflex
Sboy
Sso
SC1
SF1
SE1
Sflex-
Pae
SI
100 mM
50 µM

(b)


Sflex (SI)
Sflex + Dgal
SE1 (SI)
SE1 + Dgal
SF1 (SI)
SF1 + Dgal
SC1 (SI)
SC1 + Dgal

(c)



(d)

Figure 6. Ability of Shigella strains to emulsify hydrocarbons in the presence of salicylic acid and D-galactose. (a): This figure shows the effect of salicylic acid on the ability of strains to emulsify hydrocarbons at different concentrations; (b): effect of D-Galactose on the ability of strains to emulsify hydrocarbons at different concentrations; (c): picture showing the ability of *Shigella* to emulsify hydrocarbons in the presence or absence of D-Galactose; (d): picture showing the ability of *Shigella* to emulsify hydrocarbons in the presence or absence of Salicylic acid. Pae: *P. aeruginosa*; Sflex: *S. flexneri* M90T; Sflex-: negative control (*S. flexneri* M90T spa40-); Sboy: *S. boydii*; Sso: *S. sonnei*; SC: Clinical strain of *Shigella* spp.; SE: Environmental strain of *Shigella* spp. isolated from sewage; SF1: strain of *Shigella* spp. isolated from faecal contaminated soil; SC1: clinical strain of *Shigella* sp isolated from hospital environment.

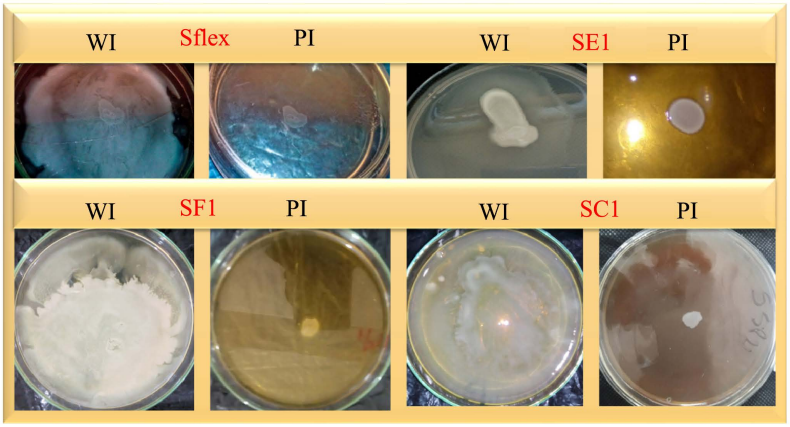


Figure 7. Swarming profile of *Shigella* sp strains in the presence of D-galactose or Salicylic Acid inhibitors. WI: without inhibitor; PI: Presence of an inhibitor's concentration; Sflex: *S. flexneri* M90T; SE1: environmental strain of *Shigella* spp. isolated from sewage; SF1: strain of *Shigella* spp. isolated from faecally contaminated soil; SC1: clinical strain of *Shigella* spp. isolated from hospital.

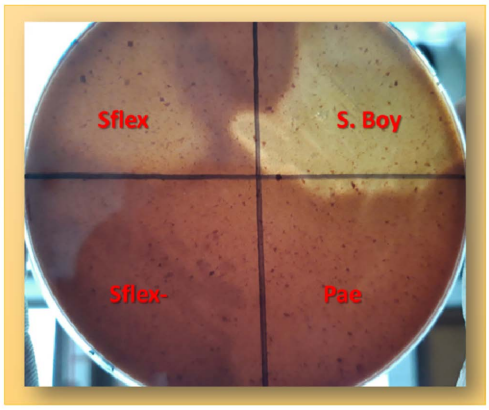
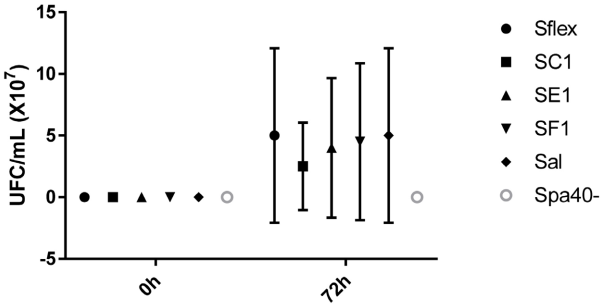
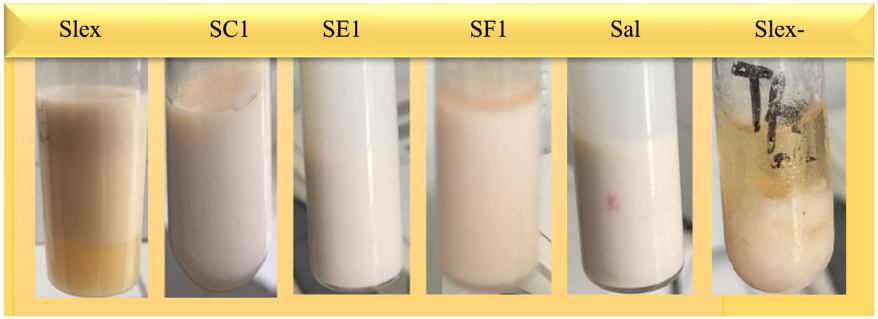


Figure 8. Appearance of *Shigella* colonies on chocolate medium + polyvitex. The presence of the clear halo reflects haemolysis around Sflex, Sboy cultures and the absence of halo around Sflex- and Pae. Sflex: *S. flexneri* 5a M90T; Sflex-: *S. flexneri* 5a M90T spa40-; Sboy: *S. boydii*. The profile was the same for all environmental strains tested.



(a)



(b)

Figure 9. Egg contamination test with *Shigella* sp isolated from the environment; (a): Bacterial enumeration of eggs contaminated with bacteria after 72 hours; (b): emulsification profile appearance of egg emulsification ability of Shigella strains used in this study. Sal: *Salmonella* spp. (positive control); Sflex: S. *flexneri* 5a M90T (reference strain); SE1: environmental strain of *Shigella* spp. isolated from sewage; SF1: strain of *Shigella* spp. isolated from faecally contaminated soil; SC1: clinical strain of *Shigella* spp.

oxidase test, and the ability of the strains to metabolize lactose, and microscopically by checking motility and biochemically.

To answer the question of whether biosurfactants are secreted during the invasion of epithelial cells by *Shigella* spp., a hydrocarbon emulsification test was

performed on the contents of eggs contaminated and uncontaminated by bacteria. Indeed, we demonstrated that the contents of the uncontaminated eggs did not emulsify the hydrocarbons, *i.e.* the emulsification index was zero. On the other hand, we obtained emulsification indices of 100% for the contaminated egg tested. The *S. flexneri* 5a M90T spa40-mutant was unable to invade the cells and therefore its contents were not emulsified (**Figure 9(a)**).

GST Pull-Down Assay and Biosurfactant-Protein Interaction Test

This test was carried out on the supernatants of cultures of *Shigella* spp that were resistant to acidic pH. We observed the appearance of a precipitate due to the action of hydrochloric acid. We collected the precipitate consisting of pellets after centrifugation at 6000 rpm. To confirm the extraction of the emulsifying agent (biosurfactant), we performed an emulsification test as an internal control. The extracted biosurfactant residue was used to study the biosurfactant and protein interaction of the translocon and tip with the biosurfactant. This present study showed that the emulsification test carried out on the GST-Ipa eluates (C, D, and B) did not lead to the formation of an emulsion, reflecting the coexistence of hydrophobic and hydrophilic phases.

4. Discussion

This work was carried out to evaluate the pathogenicity of *Shigella* spp isolated from wastewater samples, soil, and material contaminated by faeces in the city of Brazzaville. Fifty-four (54) of SES have been identified on the basis of classical microbiology techniques as belonging to the genus *Shigella*. A comparative study has been conducted between four (4) reference strains including *S. flexneri* 5a M90T, *S. boydii*, *S. sonnei*, and *S. flexneri* 5a M90T *spa40*-, SES and SCS. SES was identified on the basis of cultural characteristics of culture medium, cell morphology after staining, Gram test, cell mobility, biochemical tests (catalase and oxidase), and resistance to streptomycin. SCS were identified with API20E.

Resistance to gastric pH is an important barrier in the pathogenesis of enteric bacteria like *Shigella* due to its acidity (pH 1 - 3), which generally eliminates most bacteria that enter via the digestive tract [4]. The gastric acid resistance test performed on *Shigella* Environmental Strain (SES) and *Shigella* Clinical Strains (SCS) resulted in 38.8% (21/54) and 100% acid resistant, respectively. *Shigella* spp. Strains (SES and SCS) were ranged in a survival percentage from 11% to 93%. Since acid resistance is the criterion for pathogenicities like *Shigella* and *E. coli*. A strain of *Shigella* is supposed to be acid-resistant when the survival percentage is up to 10% [14] [15]. The SES was potentially considered pathogenic in terms of their ability to reach the colon. 33/54 strains were sensitive to acid resistance. This could be linked to the loss of the virulence plasmid pWR100 [16] [17] [18] [19] [20], the noncontribution of the lipopolysaccharide to the resistance of *Shigella* spp. to extreme acidity [21], or the absence of gene *hdeA* involved in pH resistance. Bacteria like enteropathogenic *E. coli*, *Shigella*, and *Brucella*, have been shown to possess the *hdeA* gene encoding the amphiphilic

protein HdeA which plays an important role in resistance to gastric acidity. Indeed, in *E. coli*, HdeA has, in its internal part, a hydrophobic region exposed on the surface and positively charged residues in its C-terminal part which give it a chaperone activity allowing it to resist low pH [22].

Our previous work has recently demonstrated that *Shigella* flexneri 5a M90T, *Shigella boydii*, and *Shigella sonnei* have the ability to produce biosurfactant-like Molecules (BLM). The secretion is depending on the Type Three Secretion System (T3SS). In addition, the study suggests that BLM has a bright role in the pathogenesis mechanisms of *Shigella* [8]. In bacteria like *Salmonella enteridis thyphimurium* SE 86 biosurfactants play a role in the attachment of bacteria to plant cells [23] and thus participate in its pathogenesis. The role of biosurfactants has also been pointed out and highlighted in *P. aeruginosa* cystic fibrosis [24]. The emulsification test (EI24) is allowed to demonstrate the ability of some bacteria to secrete biosurfactants. This new work has shown confirmed that *Shigella* spp isolated from the environment were all capable of emulsifying hydrocarbons with an emulsification rate of around 100%. The same profile was observed in *S. flexneri* 5a M90T, *S. boydii*, *S. sonnei*, and *P. aeruginosa* [8] [25].

In *P. aeruginosa*, biosurfactant production including lipopeptide, and rhamnolipid is under the control of three Quorum sensing (QS) regulatory pathways (LasRI, RhlRI, and PQS) [12].

By monitoring BLM production, we showed that the BLM production of clinical and environment *Shigella* sp was highly dependent on bacterial culture density. This result suggests that BLM production in *Shigella* sp could be under the control of QS. The pathway uses autoinducers (AIs) as molecular messengers. To confirm this observation, we used molecules such as D-Galactose (DGal) and Salicylic Acid (SA), which are recognized as chemical inhibitors of QS. DGal and SA have been shown to systematically prevent QS in several bacterial species including *Vibrio harveyi*, *Yersinia ruckeri*, *Sodalis praecaptivus*, *P. aeruginosa* and *Burkholderia glumae* [26] [27] [28].

In this work, we showed that with DGal 10 µM - 100 mM the growth of *Shigella* spp. was not affected. Similarly, in the presence of SA concentrations ranging from 0.5 mg/mL to 1.5 mg/mL, the growth of bacteria was not also modified. An optimum growth concentration could be seen at 1.25 mg/mL. At these standardized concentrations of D-Gal and SA, we showed that the environmental *Shigella* sp strains as well as all reference ones lose their ability to emulsify hydrocarbons and to swarm on semisolid media. This phenotype was only restored when these bacteria were grown in the absence of D-Gal and SA. Some studies have shown that SA inhibits QS by binding to the AIs receptor (LasR), thus preventing AIs to regulate the mechanisms of virulence factor expression and biofilm formation in *P. aeruginosa* [27]. Although homolog genes responsible for N-Acyl homoserine lactone (AHL) synthesis in *Shigella* spp. have not yet been found, our results suggest that a similar type of mechanism for AHL production could be possible. Since it has been shown that, some bacterial species that do not synthesize AHLs regulate QS-dependent genes in the presence of exogenous

AIs due to the presence of receptor homologs of AIs including AHLs in LuxR and LasR pathways. SdiA is an orphan LuxR-type receptor found in *Escherichia*, *Shigella*, *Salmonella*, *Klebsiella*, and *Enterobacter* genera that responds to AHL signals produced by other species and regulates genes involved in several aspects of host colonization. We believe this molecule could involve in this regulatory mechanism. For the internalization of AI, several receptors have been described including LsrB in *E. coli* and *S. typhimurium*, AgrC in *S. aureus*; TlpB in *Helicobacter* pylori and LuxP in *Vibrio* [26]. Due to the similarity of its AI transport receptors to receptors involved in sugar metabolism such as RsbB (ribose transport). These receptors could be involved in AI transport [29]. This allows us to suggest that D-galactose could competitively prevent the internalization of autoinducers (AI-2) in bacteria of the genus *Shigella*.

Eun-Ju Ryu *et al.* [28] have shown that D-galactose inhibits QS involving AI-2 and biofilm formation. This sheds light on the link between the loss of emulsifying and swarming capacity in *Shigella* spp. and the QS regulatory system.

A first approach to the involvement of QS in *Shigella* virulence had shown that QS AI-2 positively influences the expression of the VirB [30]. Indeed, assuming that QS is strongly involved in the mechanism of BLM production in bacteria of the genus *Shigella*, and that BLM is necessary for the infection pathogenicity mechanisms. This has been demonstrated in *P. aeruginosa* [31]. The communication phenomenon would lately regulate the BLM production during the infection, *i.e.* after epithelial cell invasion and during cell colonization leading to necrosis. Therefore, these observations suggest that BLM production in *Shigella* could be regulated by another mechanism dependent on the T3SS during the pre-invasive phase. BLM could be secreted under leaky secretion apparatus conditions. Later, in the pro-invasive phase, to maintain the infection, QS would have a more pronounced impact on the production of its BLM as the bacterial population increases dramatically within the colonic epithelium. Adding the fact that QS depends on bacterial cultural density, this hypothesis seems to be the most likely. However, some experimental aspects are on the way to being further demonstrated in our laboratory (**Figure 10**).

The pathogenicity of *Shigella* is determined by their ability to invade, colonize and disseminate within the colonic epithelium [32] [33]. Our study showed that after contamination of eggs, *S. flexneri*, *S. boydii* and *S. sonnei* and the 21/54 environmental *Shigella* spp. strains can invade and contaminate eggs. The counting bacteria was ranging from 2 to 5×10^7 CFU/mL after contamination. None CFU was obtained by using the mutant spa40-. Previous studies have demonstrated that the bacteria like Shigella and Salmonella could easily contaminate poultry eggs [34] [35] [36].

A previous study conducted in our laboratory suggests that biosurfactants are involved in epithelial cell adhesion and invasion [37]. We showed that the contents of contaminated eggs were able to emulsify hydrocarbons in contrast to eggs that were not contaminated with *Shigella*. This observation could confirm that biosurfactants are also secreted during the pro-invasive phase. This result

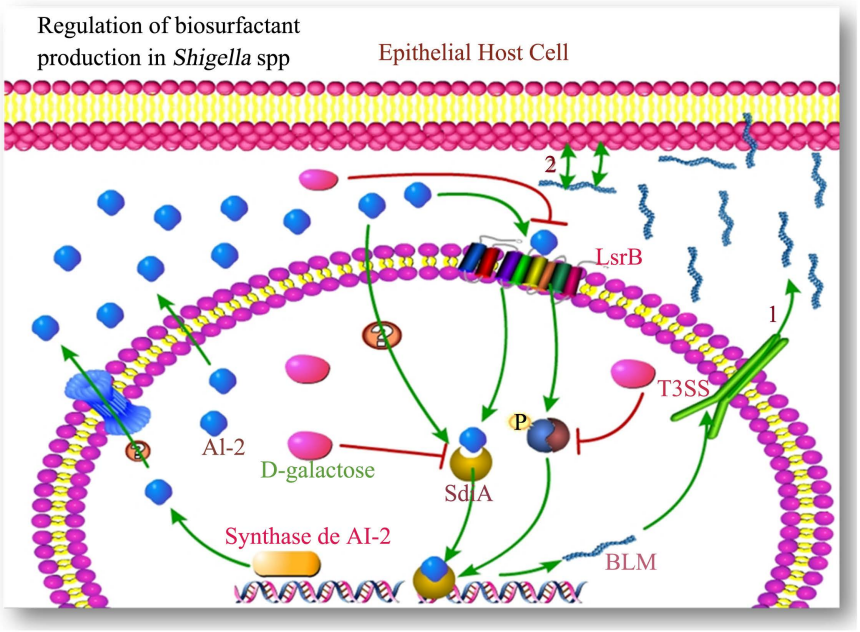


Figure 10. Model for the regulation of biosurfactant-like-molecule production by bacteria of the genus *Shigella*, proposed in this study. This model suggests two possibilities, 1 suggests the production of biosurfactants-like-molecule via the T3SS; the second way suggest (2) the BLM-basolateral membrane interaction of epithelial cells.

allows us to propose an additional model of cell invasion that also involves biosurfactants-like molecules.

Ipa B and IpaC constitute a translocon of the secretory apparatus. Both effector protein has the property of interacting with the cytoplasmic membrane of epithelial cells. IpaB is a cholesterol-binding protein [38] and binds to raft lipid motifs and interacts with integrins and CD44 [39]. These characteristics suggest that this molecule is responsible for the emulsifying capacity observed in all bacteria belonging to this genus. Moreover, in our previous study we showed that this biosurfactant would be of a protein or lipopeptide features [37].

Furthermore, the cell extract containing Gst-IpaB, IpaC, and IpaD did not emulsify the hydrocarbons either. This result allows us to rule out the hypothesis that neither IpaB, IpaC, or IpaD is responsible for emulsifying capacity. Moreover, in *Shigella* pathogenicity, the secreted BLM only targets the membrane interface, namely 1) the BLM reduces the tension at the membrane interfaces and favours the insertion of the translocon into the epithelial cell membrane and pore formation; 2) the BLM being secreted pre-invasion would also be involved in the escape from phagocytosis because once phagocytosed, the secretory apparatus emits signals that induce cell death of the macrophage as it has been reported for rhamnolipids in *P. aeruginosa* [40]. We have shown that the BLM is secreted in the pro-invasive period and at this time it is the late effectors of *Shigella* that come into play. This aspect of pathogenesis also rules out the hypothesis that translocon proteins are responsible for the emulsifying power. The *Shigella* BLM is thought to be responsible for a number of phenotypes and we be-

lieve that due to its ability to induce collective swarming movements, it is involved in cell colonization (**Figure 10**).

We also wanted to verify whether bacteria of the genus *Shigella* secrete biosurfactants during dissemination within the cells or tissues of the colonic epithelium. To this end, we experimentally demonstrated the secretion of BLM during the contamination of poultry eggs by these bacteria. The emulsification test showed that the contents of eggs contaminated with *Shigella* sp isolated from sewage and faecal contaminated soils as well as all reference strains used in this work emulsified hydrocarbons with indexes (IE24) of 100% except for the *S. flexneri* 5a M90T *spa*40-mutant. In contrast, the uncontaminated egg contents did not emulsify hydrocarbons. This result suggests that during the invasion of cells by *Shigella*, they produce and secrete biosurfactants into the extracellular medium. These biosurfactants would therefore have a major role to play in the pathogenicity of *Shigella*. This ability seems to be common to all *Shigella* species. Although this work does not discriminate between the different *Shigella* species isolated from the environment, we can say that our results confirm those obtained in a previous study [37].

Our study showed that *Shigella* strains isolated from the environment had the ability to lyse red blood cells, with the appearance of a yellow lysis hallo reflecting alpha-type haemolysis. No other *Shigella* strain showed any other type of haemolysis except the mutant which did not destroy blood cells. This can be explained by the fact that its secretory apparatus is not functional and that the effectors involved in this mechanism are not secreted into the extracellular medium [41].

Shigella are immobile bacteria, they do not have a flagellum. The movement of the bacteria within the cell is modulated by the IcsA (VirG) protein [42], which recruits and activates, via its N-terminal domain, cellular factors including the N-WASP (Wiskott-Aldrich Syndrome Protein) and the Arp2/3 complex [43]. This multi-protein complex allows actin nucleation and catalyses the elongation of an actin tail that propels *Shigella* through the cytoplasm of the host epithelial cell (**Figure 11(a)**). EPECs, *S. flexneri*, and *Salmonella* can alter the phosphorylation status and distribution of occludin and zonula occludens (ZO1), allowing the bacteria to disrupt the function of the epithelial barrier [44]. Furthermore, the role of a specific effector has not been clearly identified. It will be important to highlight this crucial interaction and to truly understand the role of tight junction modifications during *Shigella* infection. Indeed, in the light of our results, it seems more than obvious that this effector is none other than the biosurfactant secreted by *Shigella* because, as mentioned above, the surface-active properties of its molecules make them more capable of destabilising the tight junctions of cells as demonstrated with *P. aeruginosa* Rhamnolipids.

In addition, we have shown in our work that all the bacteria of the genus Shigella tested swarmed on a semi-solid medium, proof that these bacteria thanks to the production of surfactants can move. This supports the fact that one of the underlying mechanisms of *Shigella*'s intracellular spread would be the production

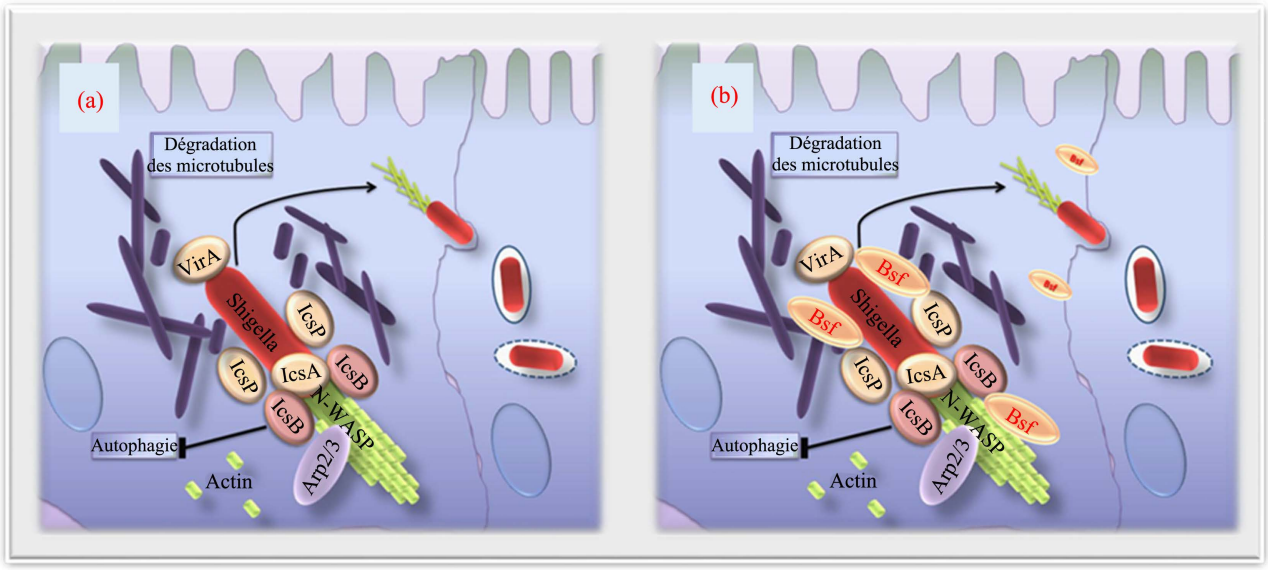


Figure 11. Model of intra- and intercellular motility of *Shigella*. (a): Classical dissemination model of *Shigella* strain in epithelial cells invasion; (b): Dissemination model involving biosurfactants-like-molecule proposed in this work.

of biosurfactants that accompany this movement. Indeed, IcsA mutants are able to enter epithelial cells with the same efficiency as the wild type. However, they are unable to move into the cell cytoplasm, form protrusions, or disseminate in a cell monolayer. This observation sufficiently demonstrates that these mechanisms are interdependent. Indeed, the recruitment of actin filaments would be the guide, the cytoskeleton the support, and the biosurfactant the sliding agent allowing the bacteria to slide, on the one hand, form protrusions on the other, but also destabilise the tight junctions/membrane, and favour the tissue diffusion of the infection (**Figure 11(b)**).

Autophagy is an intracellular host defense process that results in the lysosomal degradation of large organelles and protein complexes [45]. It is induced during stress, nutrient deprivation, bacterial or viral infection. In Shigella, the icsB gene encodes an effector protein that plays an important role in the protection mechanisms against autophagy [46] [47]. However, IcsB is not the only Shigella effector to have a direct or indirect role in activating or protecting against this process.

Kayath *et al.* [47] have shown that the ability of the IcsB protein to bind to cholesterol allows the bacterium to escape from the autophagosome formed by the host cell to destroy the bacterium. Therefore, deletion of the Cholesterol Biding Domain (CBD) significantly increases autophagosome formation. In addition, IcsB mutants were mostly unable to multiply within epithelial cells [17].

Autophagy, an intrinsic cellular catabolic system that mediates organelle turnover and cell survival under nutrient-deficient conditions, is involved in a variety of cellular biological processes, including embryonic development, cell death, and survival decisions, and the shaping of the development of the immune system. Kayath *et al.* [47], also showed that the IcsB protein interacts with

the IcsA protein to escape autophagy. It prevents the binding of Atg5, an autophagy signalling protein, to IcsA and, secondly, has a cholesterol-binding domain that induces autophagosome destruction [47]. The LC3 and Atg5 signals in the vicinity of the intracellular icsB mutant are occasionally distributed asymmetrically and observed to have accumulated at one pole of the mutant bacteria. The asymmetric distribution of the autophagic signals on the icsB mutant is reminiscent of the asymmetric distribution of VirG (IcsA) on Shigella that is essential to mediating the actin-based motility of Shigella, and raises the possibility that VirG is a target for autophagy. These observations suggest two possibilities: 1) the IcsB protein possesses a biosurfactant binding area or interaction domain that potentiates the action of this protein in vivo, 2) IcsA possesses, in addition to the IcsB interaction domain, a biosurfactant binding or interaction domain that potentiates the escape of the bacterium from autophagy (by destabilising the autophagosome) and concomitantly to the bacterial dissemination (**Figure 12**). However, the mere fact that IcsB mutants are unable to disseminate suggests that the biosurfactant is not directly responsible for autophagy escape, firstly because it is established that both IcsB and Atg5 proteins have the same IcsA/VirG binding domain, so this protective role would be paramount. Secondly, it seems more obvious that this whole multiprotein complex is required to destabilise the autophagosome (which remains to be demonstrated). However, when IcsB competes with Atg5 to bind to IcsA/VirG, the whole Biosurfactant-IcsA/VirG-IcsB

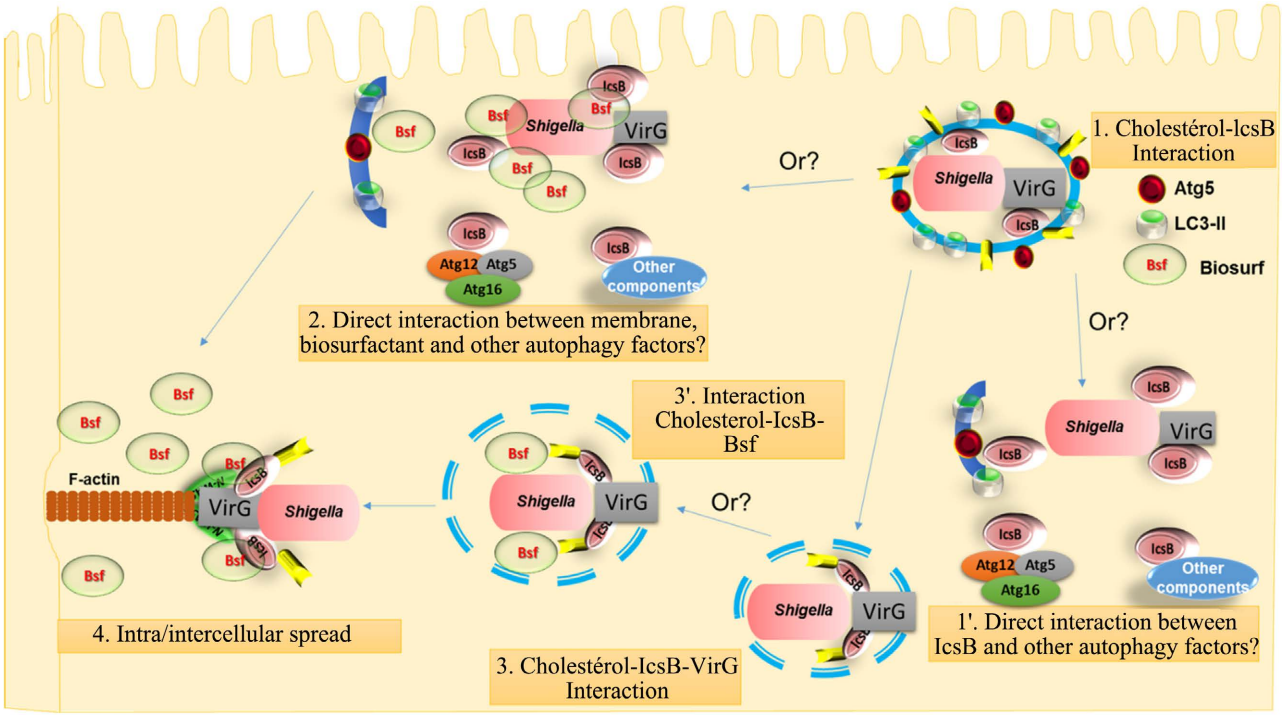


Figure 12. Modified *Shigella* autophagy escape model of Kayath *et al.* [47] involving biosurfactants. This model proposes an escape mechanism from autophagy in bacteria of the genus *Shigella*. 1. Cholesterol-IcsB Interaction; 1: Direct interaction between IcsB and other autophagy effectors; 2. Direct interaction between membrane, biosurfactant and other autophagy factors; 3. Cholesterol-IcsB-VirG interaction; 3. Interaction Cholesterol-IcsB-VirG-Bsf; 4. Intra/Intercellular spread.

complex interacts with the membrane via the CBD of IcsB and the biosurfactant interface which results in the destabilisation of the membrane, and then the biosurfactant assists in the dissemination as proposed above. It has recently been shown that the *Shigella* surface protein IcsA, in addition to its role in actin-based motility, acts as a host cell adhesin by one or more unknown mechanisms, which the authors were unable to elucidate [48]. We believe that this would be an interaction with a surfactant, which would be of a protein nature as demonstrated during his thesis work.

5. Conclusion

The aim of the present study was to assess the pathogenicity of circulating *Shigella* sp strains isolated from the environment from source points, wastewater, and faecally contaminated soil and to contribute to the understanding of the mechanism of invasion of epithelial cells. *Shigella* Environmental strains were able to produce and secrete BLM in the extracellular medium. BLM during the pre-, pro-, and post-invasion phases, the production and secretion of biosurfactants could be regulated by the T3SS assembly during the pre- and pro-invasion phases. This work highlighted that during the post-invasion phase the production and secretion of these molecules are regulated by Quorum sensing pathways. This study also showed that bacteria of the genus *Shigella* isolated from the environment possessed a beta-type haemolytic activity, indicating their capacity to invade and colonize cells. This work resulted in the proposal of a model of cell invasion by *Shigella* in light of the results obtained during our research work.

Acknowledgements

The authors wish to express their profound gratitude to Mr. Bantsimba Malonga Loïc Marlye Djesone, Mr. Kapéndé Chrislen Mavy, Koyondopa, and Mr. Koyandakpa Aristote for technical support.

Data Availability

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

Conflicts of Interest

The authors declare that there are no conflicts of interest.

References

[1] Schroeder, G.N. and Hubert, H. (2008) Molecular Pathogenesis of *Shigella* Spp.: Controlling Host Cell Signaling, Invasion, and Death by Type III Secretion. *Clinical Microbiology Reviews*, **21**, 134-156. https://doi.org/10.1128/CMR.00032-07

[2] Havelaar, A.H., Kirk, M.D., Torgerson, P.R., Gibb, H.J., Hald, T., Lake, R.J., *et al.* (2015) World Health Organisation Global Estimates and Regional Comparisons of the Burden of Foodborne Disease in 2010. *PLoS Medicine*, **12**, Article ID: e1001923. https://doi.org/10.1371/journal.pmed.1001923

[3] Kotloff, K.L., Riddle, M.S., Platts-Mills, J.A., Pavlinac, P. and Zaidi, A.K.M. (2018) Shigellosis. *The Lancet*, **391**, 801-812. https://doi.org/10.1016/S0140-6736(17)33296-8

[4] Zhang, J., Wang, F., Jin, H., Hu, J., Yuan, Z., Shi, W., Yang, X., Meng, J. and Xu, X. (2015) Laboratory Monitoring of Bacterial Gastroenteric Pathogens *Salmonella* and *Shigella* in Shanghai, China 2006-2012. *Epidemiology and Infection*, **143**, 478-485. https://doi.org/10.1017/S0950268814001162

[5] Smith, D.K., Kassam, T., Singh, B. and Elliott, J.F. (1992) Escherichia Coli Has Two Homologous Glutamate Decarboxylase Genes That Map to Distinct Loci. *Journal of Bacteriology*, **174**, 5820-5826. https://doi.org/10.1128/jb.174.18.5820-5826.1992

[6] Kayath, A.C., Hussey, S., Hajjami, N.E., Nagra, K., Philpott, D. and Allaoui, A. (2010) Escape of Intracellular *Shigella* from Autophagy Requires Binding to Cholesterol through the Type III Effector, IcsB. *Microbes and Infection*, **12**, 956-966. https://doi.org/10.1016/j.micinf.2010.06.006

[7] Botteaux, A., Kayath, C.A., Page, A.-L., Jouihri, N., Sani, M., Boekema, E., *et al.* (2010) The 33 Carboxyl-Terminal Residues of Spa40 Orchestrate the Multi-Step Assembly Process of the Type III Secretion Needle Complex in *Shigella flexneri*. *Microbiology*, **156**, 2807-2817. https://doi.org/10.1099/mic.0.039651-0

[8] Kinouani Kinavouidi, D.J., Kayath, C.A. and Nguimbi, E. (2020) Invasion of Epithelial Cells Is Correlated with Secretion of Biosurfactant via the Type 3 Secretion System (T3SS) of *Shigella flexneri*. *Journal of Pathogens*, **2020**, Article ID: 3062821. https://doi.org/10.1155/2020/3062821

[9] Rosenberg, M., Gutnick, D. and Rosenberg, E. (1980) Adherence of Bacteria to Hydrocarbons: A Simple Method for Measuring Cell-Surface Hydrphobicity. *FEMS Microbiology Letters*, **9**, 29-33. https://doi.org/10.1111/j.1574-6968.1980.tb05599.x

[10] Baris Gökalsin, D.B. and Nüzhet, C.S. (2019) *Pseudomonas aeruginosa* Quorum Sensing and Biofilm Inhibition. In: Tommonaro, G., Ed., *Quorum Sensing: Molecular Mechanism and Biotechnological Application*, Elsevier Inc., Amsterdam, 227-256. https://doi.org/10.1016/B978-0-12-814905-8.00009-5

[11] Kearns, D.B. (2010) A Field Guide to Bacterial Swarming Motility. *Nature Reviews Microbiology*, **8**, 634-644. https://doi.org/10.1038/nrmicro2405

[12] Déziel, E., Lépine, F., Milot, S. and Villemur, R. (2003) *rhlA* Is Required for the Production of a Novel Biosurfactant Promoting Swarming Motility in *Pseudomonas aeruginosa*: 3-(3-hydroxyalkanoyloxy)alkanoic Acids (HAAs), the Precursors of Rhamnolipids. *Microbiology*, **149**, 2005-2013. https://doi.org/10.1099/mic.0.26154-0

[13] Sharma, M. and Arnaud, S.K. (2002) Swarming: A Coordinated Bacterial Activity. *Current Science*, **83**, 707-715.

[14] Zhao, B. and Houry, W.A. (2010) Acid Stress Response in Enteropathogenic Gammaproteobacteria: An Aptitude for Survival. *Biochemistry and Cell Biology*, **88**, 301-314. https://doi.org/10.1139/O09-182

[15] Gorden, J. and Small, P.L. (1993) Acid Resistance in Enteric Bacteria. *Infection and Immunity*, **61**, 364-367. https://doi.org/10.1128/iai.61.1.364-367.1993

[16] Allaoui, A., Menard, R., Sansonetti, P.J. and Parsot, C. (1993) Characterization of the *Shigella flexneri* ipgD and ipgF Genes, Which Are Located in the Proximal Part of the MXI Locus. *Infection and Immunity*, **61**, 1707-1714. https://doi.org/10.1128/iai.61.5.1707-1714.1993

[17] Allaoui, A., Mounier, J., Prevost, M.C., Sansonetti, P.J. and Parsot, C. (1992) *icsB*: A *Shigella flexneri* Virulence Gene Necessary for the Lysis of Protrusions during Intercellular Spread. *Molecular Microbiology*, **6**, 1605-1616.

https://doi.org/10.1111/j.1365-2958.1992.tb00885.x

[18] Allaoui, A., Sansonetti, P.J., Menard, R., Barzu, S., Mounier, J., Phalipon, A., *et al.* (1995) MxiG, a Membrane Protein Required for Secretion of *Shigella* spp. Ipa Invasins: Involvement in Entry into Epithelial Cells and in Intercellular Dissemination. *Molecular Microbiology*, **17**, 461-470. https://doi.org/10.1111/j.1365-2958.1995.mmi_17030461.x

[19] Allaoui, A., Sansonetti, P.J. and Parsot, C. (1992) MxiJ, a Lipoprotein Involved in Secretion of *Shigella* Ipa Invasins, Is Homologous to YscJ, a Secretion Factor of the Yersinia Yop Proteins. *Journal of Bacteriology*, **174**, 7661-7669. https://doi.org/10.1128/jb.174.23.7661-7669.1992

[20] Allaoui, A., Sansonetti, P.J. and Parsot, C. (1993) MxiD, an Outer Membrane Protein Necessary for the Secretion of the *Shigella flexneri* lpa Invasins. *Molecular Microbiology*, **7**, 59-68. https://doi.org/10.1111/j.1365-2958.1993.tb01097.x

[21] Martinic, M., Hoare, A., Contreras, I. and Alvarez, S.A. (2011) Contribution of the Lipopolysaccharide to Resistance of *Shigella flexneri* 2a to Extreme Acidity. *PLoS ONE*, **6**, Article ID: e25557. https://doi.org/10.1371/journal.pone.0025557

[22] Wu, Y.E., Hong, W., Liu, C., Zhang, L. and Chang, Z. (2008) Conserved Amphiphilic Feature Is Essential for Periplasmic Chaperone HdeA to Support Acid Resistance in Enteric Bacteria. *Biochemical Journal*, **412**, 389-397. https://doi.org/10.1042/BJ20071682

[23] Machado, T., Malheiros, P., Brandelli, A. and Tondo, E.C. (2010) Évaluation de la résistance de la Salmonelle à l'action des désinfectants acide peracético, quaternário de l'aminé et hypoclorite de sodium. *Revista do Instituto Adolfo Lutz*, **69**, 475-481.

[24] Satpute, S.K., Banpurkar, A.G., Dhakephalkar, P.K., Banat, I.M. and Chopade, B.A. (2010) Methods for Investigating Biosurfactants and Bioemulsifiers: A Review. *Critical Reviews in Biotechnology*, **30**, 127-144. https://doi.org/10.3109/07388550903427280

[25] Yerpude, S.R.K. (2019) Emulsifying and Flocculating Performances of Exopolysacharides Produced by Bacillus Subtilis Strains and *Shigella flexneri*. *International Journal of Research and Analytical Reviews*, **5**, 453-456.

[26] Guo, M., Gamby, S., Zheng, Y. and Sintim, H.O. (2013) Small Molecule Inhibitors of AI-2 Signaling in Bacteria: State-of-the-Art and Future Perspectives for anti-Quorum Sensing Agents. *International Journal of Molecular Sciences*, **14**, 17694-17728. https://doi.org/10.3390/ijms140917694

[27] El-Mowafy, S.A., Abd El Galil, K.H., El-Messery, S.M. and Shaaban, M.I. (2014) Aspirin Is an Efficient Inhibitor of Quorum Sensing, Virulence and Toxins in *Pseudomonas aeruginosa*. *Microbial Pathogenesis*, **74**, 25-32. https://doi.org/10.1016/j.micpath.2014.07.008

[28] Ryu, E.-J., Sim, J.H., Sim, J., Lee, J. and Choi, B.-K. (2016) D-Galactose as an Autoinducer 2 Inhibitor to Control the Biofilm Formation of Periodontopathogens. *Journal of Microbiology*, **54**, 632-637. https://doi.org/10.1007/s12275-016-6345-8

[29] Reen, F.J., Holcombe, L.J., McSweeney, C.M., McGlacken, G.P., Morrissey, J.P. and O'Gara, F. (2013) The *Pseudomonas* Quinolone Signal (PQS), and Its Precursor HHQ, Modulate Interspecies and Interkingdom Behaviour. *FEMS Microbiology Ecology*, **77**, 413-428. https://doi.org/10.1111/j.1574-6941.2011.01121.x

[30] Papenfort, K. and Bassler, B. (2019) Quorum-Sensing Signal-Response Systems in Gram Negative Bacteria. *Nature Reviews Microbiology*, **14**, 576-588. https://doi.org/10.1038/nrmicro.2016.89

[31] Williams, P. and Cámara, M. (2009) Quorum Sensing and Environmental Adapta-

tion in *Pseudomonas aeruginosa*: A Tale of Regulatory Networks and Multifunctional Signal Molecules. *Current Opinion in Microbiology*, **12**, 182-191. https://doi.org/10.1016/j.mib.2009.01.005

[32] Schnupf, P. and Sansonetti, P.J. (2019) *Shigella* Pathogenesis: New Insights through Advanced Methodologies. *Microbiology Spectrum*, **7**. https://doi.org/10.1128/microbiolspec.BAI-0023-2019

[33] Killackey, S.A., Sorbara, M.T. and Girardin, S.E. (2016) Cellular Aspects of *Shigella* Pathogenesis: Focus on the Manipulation of Host Cell Processes. *Frontiers in Cellular and Infection Microbiology*, **6**, Article No. 38. https://doi.org/10.3389/fcimb.2016.00038

[34] Gantois, I., Ducatelle, R., Pasmans, F., Haesebrouck, F., Gast, R., Humphrey, T.J., *et al.* (2009) Mechanisms of Egg Contamination by Salmonella Enteritidis. *FEMS Microbiology Reviews*, **33**, 718-738. https://doi.org/10.1111/j.1574-6976.2008.00161.x

[35] Muleta, D. and Ashenafi, M. (2001) Salmonella, Shigella and Growth Potential of Other Food-Borne Pathogens in Ethiopian Street Vended Foods. *East African Medical Journal*, **78**, 576-580. https://doi.org/10.4314/eamj.v78i11.8946

[36] Whiley, H. and Ross, K. (2015) Salmonella and Eggs: From Production to Plate. *International Journal of Environmental Research and Public Health*, **12**, 2543-2556. https://doi.org/10.3390/ijerph120302543

[37] Kinavouidi, D.J.K., Aimé Kayath, C. and Nguimbi, E. (2020) Invasion of Epithelial Cells Are Correlated with Secretion of Biosurfactant via the 2 Type 3 Secretion System (SST3) of *Shigella flexneri*. *Journal of Pathogens*, **2020**, Article ID 3062821. https://doi.org/10.1155/2020/3062821

[38] Epler, C., Dickenson, N.E., Olive, A.J., Picking, W.L. and Picking, W.D. (2009) Liposomes Recruit IpaC to the *Shigella flexneri* Type III Secretion Apparatus Needle as a Final Step in Secretion Induction. *Infection and Immunity*, **77**, 2754-2761. https://doi.org/10.1128/IAI.00190-09

[39] Skoudy, A., Mounier, J., Aruffo, A., Ohayon, H., Gounon, P., Sansonetti, P., *et al.* (2000) CD44 Binds to the *Shigella* IpaB Protein and Participates in Bacterial Invasion of Epithelial Cells. *Cellular Microbiology*, **2**, 19-33. https://doi.org/10.1046/j.1462-5822.2000.00028.x

[40] McClure, C. and Schiller, N. (1992) Effects of *Pseudomonas aeruginosa* Rhamnolipids on Human Monocyte Derived Macrophages. *Journal of Leukocyte Biology*, **51**, 97-102. https://doi.org/10.1002/jlb.51.2.97

[41] Picking, W.L., Nishioka, H., Hearn, P.D., Aaron Baxter, M., Harrington, A.T., Blocker, A. and Picking, W.D. (2005) IpaD of *Shigella flexneri* Is Independently Required for Regulation of Ipa Protein Secretion and Efficient Insertion of IpaB and IpaC into Host Membranes. *Infection and Immunity*, **73**, 1432-1440. https://doi.org/10.1128/IAI.73.3.1432-1440.2005

[42] Bernardini, M.L., Mounier, J., d'Hauteville, H., Coquis-Rondon, M. and Sansonetti, P.J. (1989) Identification of icsA, a Plasmid Locus of *Shigella flexneri* That Governs Bacterial Intra- and Intercellular Spread through Interaction with F-Actin. *Proceedings of the National Academy of Sciences of the United States of America*, **86**, 3867-3871. https://doi.org/10.1073/pnas.86.10.3867

[43] Suzuki, T., Mimuro, H., Suetsugu, S., Miki, H., Takenawa, T. and Sasakawa, C. (2002) Neural Wiskott-Aldrich Syndrome Protein (N-WASP) Is the Specific ligand for *Shigella* VirG among the WASP Family and Determines The Host Cell Type Allowing Actin-Based Spreading. *Cellular Microbiology*, **4**, 223-233.

https://doi.org/10.1046/j.1462-5822.2002.00185.x

[44] Sakaguchi, T., Köhler, H., Gu, X., McCormick, B.A. and Reinecker, H.-C. (2002) *Shigella flexneri* Regulates Tight Junction-Associated Proteins in Human Intestinal Epithelial Cells. *Cellular Microbiology*, **4**, 367-381. https://doi.org/10.1046/j.1462-5822.2002.00197.x

[45] Deretic, V. and Levine, B. (2009) Autophagy, Immunity, and Microbial Adaptations. *Cell Host Microbe*, **5**, 527-549. https://doi.org/10.1016/j.chom.2009.05.016

[46] Cullinane, M., Gong, L., Li, X., Lazar-Adler, N., Tra, T., Wolvetang, E., Prescott, M., Boyce, J.D., Devenish, R.J. and Adler, B. (2008) Stimulation of Autophagy Suppresses the Intracellular Survival of *Burkholderia pseudomallei* in Mammalian Cell lines. *Autophagy*, **4**, 744-753. https://doi.org/10.4161/auto.6246

[47] Kayath, C.A., Hussey, S., El hajjami, N., Nagra, K., Philpott, D. and Allaoui, A. (2010) Escape of Intracellular Shigella from Autophagy Requires Binding to Cholesterol through the Type III Effector, IcsB. *Microbes and Infection*, **12**, 956-966.

[48] Qin, J., Doyle, M.T., Tran, E.N.H. and Morona, R. (2020) The Virulence Domain of *Shigella* IcsA Contains a Subregion with Specific Host Cell Adhesion Function. *PLoS ONE*, **15**, Article ID: e0227425. https://doi.org/10.1371/journal.pone.0227425