The Occurrence and resistance of Shigella flexneri *CECT4804 to acid stress: Impact of acid stress* in vitro *and* in vivo

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Adhesion,
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Viability.

Summary

The ability to maintain intra-cellular pH is crucial for many microbes mainly the enterobacteria to survive in diverse environments, particularly those that undergo fluctuations in pH. In this context, the growth and survival of *S. flexneri* at different acid pH values were evaluated to explain the response strategies involved in the adaptation of *Shigella flexneri* CECT4804 in acid stress conditions. Furthermore, the capacity of this strain to produce slime on Congo Red Agar, biofilm formation on polystyrene plate and hydrophobicity are reported. In addition, the modification of the membrane fatty acids profiles has been studied. Moreover, an infection with the stressed strain was realized on rats in rates and examined for their toxicity in intestine tissue. The obtained results show that the strain survival is strongly influenced by acidity. Indeed, the stressed and unstressed strains became biofilm positive after acid stress. A significant increase in the hydrophobicity percentage compared to the values found under normal conditions, is also noticed. For the membrane fatty acids analysis, the acidity induces several modifications in the membrane composition. After the infection, the gravest lesion was registered in the intestine of rats administered with the bacteria stressed at the lowest pH.

Introduction

Shigella sp. is accredited as an enteric pathogen responsible for shigellosis, know with the name of *bacillary dysentery* or *Shigella dysentery* (Niyogi, 2005). These entero-pathogens induce significant human morbidity and mortality especially in low- and middle-income country due to diarrhea disease. In 2015, diarrhea is arranged as the ninth cause of death in general and the fourth cause of children death under five years-old (Hosangadi *et al.* 2018; Troeger *et al.* 2017).

Shigella sp. is one of the known borne-food pathogens (Chan and Blaschek, 2005; Warren *et al.* 2006). The strain is Gram negative, facultatively anaerobic optional, immobile smooth and with regular limits. It is an intracellular pathogen belongs

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to the family *Enterobacteriaceae*. The genus is divided into four species: *Shigella dysentery, Shigella flexneri*, *Shigella boydii, Shigella sonnei*. They are found in water, vegetables, and in many other food. *S. flexneri* is the most isolated strain because it is the major cause of diarrhea among the four species of *Shigella* (Casabonne *et al.* 2016; Zhao *et al.* 2017). These bacteria colonize the intestine tract of the host organism in order to cause an infection. Commonly, the main *Shigella* infection phases are adhesion, intracellular replication and invasion (Livio *et al.* 2014; Saeed *et al.* 2015).

Intra-cellular pathogens like *Shigella*, upon ingestion, depend on various host conditions inside the host environment (temperature, bile salt, nutrient limitation, pH, ect… (Fang *et al.* 2016; Foster, 1999; Nickerson *et al.* 2017). The acid pH or the acid stress is also naturally found in several foods, due to the use of weak organic acids or short fatty chain as a food preservative and also in the fermenting acidified fecal material (Álvarez-Ordóñez *et al.* 2010). The ability to maintain intracellular pH is crucial for enteric bacteria to survive in diverse environments, especially those that undergo fluctuation in pH. Thus, to survive in these changing environments, bacteria have developed various systems that not only cause stresses but also trigger appropriate responses that allow survival and propagation under these conditions. In this context, several researches demonstrated that intracellular pathogens as *Shigella* can use various defenses mechanisms to cope with harsh conditions Ggoh *et al.* 2011; Jennison and Verma, 2007; Ramos-Morales, 2012; Zhao and Houry, 2010). Different studies have mentioned that these stressful conditions induced responses such as desiccation by modifying hydrophobic state, its physiology and the biofilm production ability (Ellafi *et al.* 2012; Lagha *et al.* 2012), as well as changes in cell surface proprieties such as the alteration of the membrane fluidity by fatty acids composition (Haddaji *et al.* 2017; Lagha *et al.* 2015).The main objective of this research is (1) to study the effects of the acid stress on the intracellular pathogens' survival and on the membrane fatty acid composition of *S. flexneri* and (2) to demonstrate the correlation between changes of hydrophobicity and adhesion biofilm forming under stress condition.

Materials and methods

Bacterial strain and growth conditions

The *S. flexneri* (CECT 4804) strain used in this study, is from the laboratory collection. It was stored in Luria-Bertani broth (LB) and supplemented with glycerol (25%, v/v) at -80°C. For the experiments, cells' bacteria were grown in LB broth, at 37°C, for 24h.

Stress treatment

For acid stress, LB microcosms (100 mL) were prepared at final pH of 2.5, 3 and 3.5; [the pH was adjusted by hydrochloric acid (HCl)] and autoclaved (115 °C for 15 min). Other microcosms, containing a fresh lemon juice (pH=3.56) with LB, were prepared with distilled water and sterilized trough 0.22 µm microfiltre. The microcosms (100 mL) were inoculated with bacterial suspension with an optical density of 0.6 at 600 nm and then incubated at 37°C, for 2h.

MTT colorimetric assay

The monitoring viability of the microcosms was performed by colorimetric assay. This method is based on the tetrazolium salt (3-[4.5 dimethylthiazol -2-yl]-2.5-diphenyltetrazolium bromide (MTT)) reduction, which was initially yellow, then to a formazan salt (violet color) which is soluble by the metabolically active cells. One hundred microliter of MTT solution (1 mg/mL MTT in phosphate buffer salt and sterilized through 0.22 µm microfiltre) was added to 900 µl of each of the microcosms. After 2h of incubation in dark and at room temperature, the cells were lysed with a lyses solution (HCl + isopropanol) and incubated at 37°C for 1h. Finally, formazan production was quantified by photometry at 570 nm. Viability was determined in each microcosm during the inoculation, as the following: after 2h, 24h, and 48h.

Adherence assay

Qualitative adherence of stressed and controlled *S. flexneri CECT4804* was studied by culturing the strains on Congo Red Agar (CRA) made by mixing 0.8 of congo red with 36 g saccarose (Sigma) in 1l brain and heart infusion agar (BHI) Sigma). *Shigella* strain was inoculated onto the surface of CRA plates and incubated at 37 °C under aerobic conditions for 24h and followed overnight at room temperature. Slime bacteria producers appeared as black colonies, whereas non-slime ones were un-pigmented (Blanco *et al.* 2005; Chaieb *et al.* 2007).

For semi-quantitative adherence, bacteria were incubated in Tryptic Soy Broth (TSB) at 37°C, for 24h. The broths were subsequently diluted (1:100) in TSB and supplemented with 2% glucose (w/v). Two hundred microliter of bacterial suspension were transferred into 96 polystyrene ELISA plate U (Nunclon, Nunc, Roskilde, Denmark). A sterile TSB broth was used as a negative control. The plate was incubated for 24h at 37°C, rinsed three times with distilled water in order to remove the non-adherent bacteria in dry air. Then, the adherent bacteria were fixed with 95% ethanol solution and were stained with crystal violet solution (1%)

(Merk) for 5 min. The exceeded dye was removed and then washed three times with 300µl of distilled water. The plate was emptied and dried with a flow of air (Abdallah *et al*. 2009). The optical density of each sample was measured at 570 nm using an ELISA reader (automated Multiskan reader, MNOP). Each experiment was repeated three times. The strains' ability to form a biofilm is classified into three categories strongly-adherent (OD_{570}>1) weakly-adherent (0.1<OD_{570}<1) and non-adherent (OD_{570}<0.1) (Chaieb *et al*., 2007).

Cell surface hydrophobicity

Hydrophobicity was measured by the hexadecane partitioning method (Loosdrecht *et al.* 1987). Bacterial cells (control and stressed) were grown overnight in LB at different pH values (2.5, 3, 3.5) and in lemon juice, and were washed with the Phosphate Buffer Saline (PBS) and re-suspended in 4 mL of PBS. Then, the absorbance at OD_{540} was determined. One mL of hexadecane was added to each cell suspension and was equilibrated during 10 min.

Each suspension was re-incubated at 37°C for 30 min. The aqueous layer was removed and aerated to remove all traces of hexadecane, and the absorbance of hexadecane-extracted PBS blank was measured. The ratio of absorbance of the hexadecane-extracted sample indicates the hydrophobicity index (IH) compared to the absorbance of the sample before extraction.

$$IH= ((DO_1-DO_2)/DO_1) *100.$$

DO_1: absorbance of the hexadecane-extracted sample

DO_2: absorbance of the sample after extraction

Analysis of fatty acids

In order to analyze the total cellular fatty acids (FA), the control and stressed cells were recovered by centrifugation (4000 rpm for 15 min), washed three times with distilled water and pre-treated respecting the MIDI protocols (Haddaji *et al.* 2017; Sasser, 1990). Next, the final extracts were analyzed by gas chromatography with a HP-Innowax column (30 m × 0.25 mm) under the following conditions: flame ionization detector temperature at 280 °C, carrier gas was N_2 at 1 mL/min, injector temperature 270 °C, oven temperature programmed from 130 to 230 °C, using a Hewlett-Packard HP 5890 capillary gas chromatograph linked to an HP Chemstation integrator. The identification of fatty acid methyl esters was performed by external standards (all purchased from Sigma Chemical Co.) (Loosdrecht *et al.* 1987) under the same processes of manipulation as the analyzed biological samples. A known quantity of heneicosanoic acid methyl ester ($C_{21:0}$) was used as an internal injection standard. The values of fatty acids are presented as area percentage of total fatty acids. Total saturated fatty acids (SFA), total unsaturated fatty acids (UFA), and total cyclic fatty acids (CFA) are used to determine the differences between membrane fatty acids of *S. flexneri* (CECT4804) cells grown under the different conditions. The UFA/SFA ratio is used as an indirect indicator of the membrane fluidity (Lagha *et al*. 2012).

Animals and treatment

Male Wistar rats of the same age with an initial average weight about 100g were used.

The breeding of these rats has been done at the « animal sector » of the Faculty of Sciences of Gafsa, Gafsa, Tunisia. The temperature is steadily maintained at nearly 23 ± 2 °C with a regular alternance of 12 hours light and 12 hours obscurity and 55% humidity.

The rats were fed a commercial pelleted chow from SNA Sfax (Tunisia) and with drinking water ad libitum. Rats were cared using the Tunisian code of practice for the Care and Use of Animals for Scientific Purposes together with the European convention for the protection of vertebrate animals used for experimental and other scientific purposes (Council of Europe No 123, Strasbourg, 1985). All animal experiments were done according to the consent of the Medical Ethical Committee for the Care and Use of Laboratory Animals of Pasteur Institute of Tunis, Tunisia (approval number: FST/LNFP/Pro 152012). After 2 weeks of acclimatization, the animals were randomly divided into seven groups (6 animals each):

Group I (normal control group), in which the rats were orally administered saline water.

Group II, in which the rats were administered orally daily broth LB (0.5 mL).

Group III, in which the rats were orally administered daily bacteria (5×10^8 colony-forming units (CFU)) (0.5 mL).

Group IV, in which the rats were administered bacteria (5×10^8 CFU) following pH stress (pH=3.5) (0.5 mL).

Group V, in which the rats were administered bacteria (5×10^8 CFU) following pH stress (pH=3) (0.5 mL).

Group VI, in which the rats were administered bacteria (5×10^8 CFU) following pH stress (pH=2.5) (0.5 mL).

Group VII, in which the rats were administered bacteria (5×10^8 CFU) added with lemon juice (pH=3.56) (0.5 mL).

Histopathological findings

All groups underwent a 7-day treatment administered *via* gavage. At the end of the last day of treatment, rats were sacrificed through decapitation under ether anesthesia following an overnight fast. The intestines were carefully collected, weighed, and rinsed in ice-cold saline to eliminate blood residues. Subsequently, the tissues were preserved in a 4% formalin solution and promptly subjected to histopathological examinations using the paraffin technique (Ellafi *et al.*, 2023).The criteria to grade the intestinal histopathological changes were the following (Han *et al.* 2014): Score 0, no evident pathological changes; score 1–3, mild injury characterized by slight edema and a decrease in the number of mucous epithelial cells; score 4–5, moderate injury characterized by inflammatory cell infiltration, congestion, cell apoptosis and necrosis; score 6–10, severe injury characterized by massive inflammatory cell infiltration, severe hemorrhage and congestion, evident edema, coagulation necrosis and focal necrosis.

Statistical analysis

Statistical analyses were performed by SPSS 13.0 software. The results were examined by the Friedman test, followed by the Wilcoxon signed ranks test. A value of P <0.05 was considered significant**.**

Results

Effect of acid stress on viability

The survival of *S. flexneri* (CECT4804) in LB broth at different pH values (2.5, 3, 3.22 (lemon juice), 3.5, and 7) was studied.Results demonstrated that the strain survival is strongly influenced by acidity especially at pH 2.5. Also, a significant difference in the cultivability, under the acid stress effect, was noticed (Figure 1).

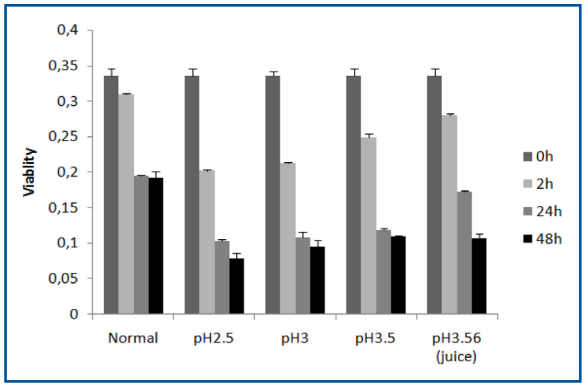

Figure 1. *Growth of* Shigella flexneri *CECT4804 as a function of pH within 0 to 48h of incubation at 37°C*

Biofilm production by *S. flexneri* (CECT 4804) under acid stress

The result of the adhesion test shows that *S. flexeneri* (CECT 4804) in the normal state develops white colonies on CRA (Figure 2A). At the contrary, under stress condition this strain develops a medium brown colony which means that it produces biofilm slides (Figure 2B). The quantitative analysis of adhesion to polystyrene ELISA plates showed that pH can significantly influence *S. flexneri* (CECT 4804) ability to form biofilm. Table I presents the results of optical density (OD_{570}) after 24 h of culture in TSB broth at different pH [2.5, 3, 3.22 (lemon juice), 3.5, and 7]. In the unstressed case, this strain remained unable to form the biofilm and therefore it is called "negative biofilm strain" (OD_{570} <0.1). However, they became "strongly biofilm positive strains" (DO_{570}> 1) beginning from the pH rate of 3.

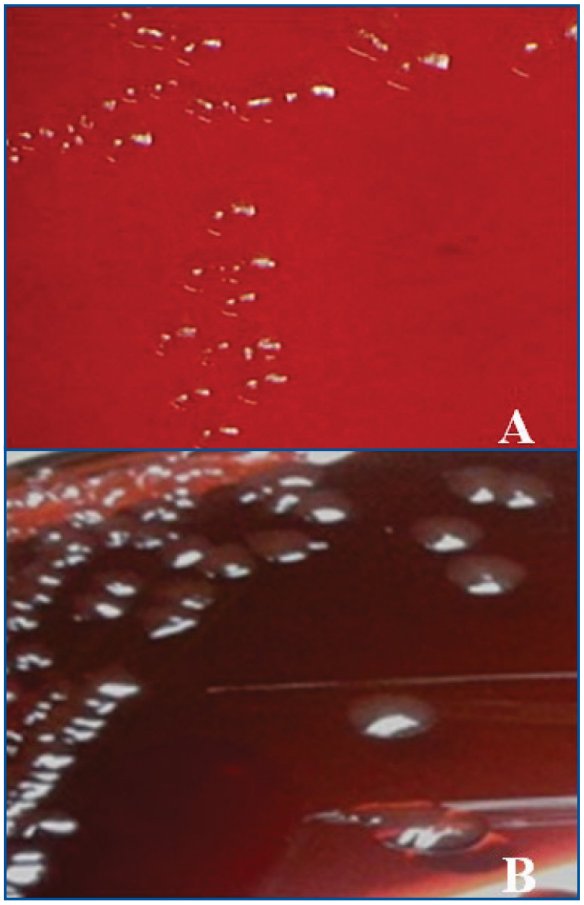

Figure 2. *Appearance of* Shigella flexneri *CECT4804 colonies on Congo Red medium before: (A) slime strain (-) and after stress conditions (B) slime strain (+).*

Table I. *Effect of pH on the Biofilm formation by* Shigella flexneri *CECT4804*

SF	Normal	pH=2.5	pH=3	pH=3.5	pH_{juice}=3.56
OD_{570nm}	0,023±0,004	0,098*±0,007	0,113*±0,012	0,129*±0,031	0,135*±0,011

*: *P*<0.05

Effect of stress on the surface hydrophobicity

Bacteria affinity with solvent was performed to evaluate the surface properties of the tested strain after acid stress. *S. flexeneri* incubation on acidic medium had a significant effect on the hydrophobicity of the cell surface of the bacterium. In the normal state, the affinity of *S. flexeneri* with n-hexadecan suggests a hydrophilic character. Depending on the acidity, we noticed a significant increase in the percentage of hydrophobicity ($P < 0.05$) compared to the values found under normal conditions (Table III).

Table II. *Hydrophobicity (% ± SD) results for normal and stressed strains of* Sigella flexneri *CECT4804*

SF	Normal	pH=2.5	pH=3	pH=3.5	pHjuice=3.56
IH	14,41±1,78	80*±2,27	74,30*±3,7	79,23*±2,47	72,99*±1,5

*: $P<0.05$

Table III. *Fatty acids composition of* Shigella flexneri *CECT4804 under stressed conditions*

pH	Fatty acid (%)																			
	C10:0	C12:0	C14:0	C14:1	C15:0 iso	C15:0 antieso	C16:1n7	C16:1n5	C16 :0	C17:0	C17:0 iso	C17:0 antieso	C17:1	C18 :0	C18:1n9	C18:1n7	C18:2n6	C18:3n6	C20:0	C20:1n9
Normal	1,09	0,33	0,12	0,12	0,04	0,53	4,59	5,61	35,52	0,45	0,63	0,32	0,59	0,56	1,88	24,64	20,04	1,43	0,17	1,25
pH 3.5	0,95	0,42	0,12	0	0,32	0,2	1,33	0,71	43,63	0,65	0,88	0,13	0,24	0,86	0,71	2,47	39,88	1,26	0,43	4,7
pH 3.56	1,53	0,4	0,12	0,12	0,44	0,45	4,82	6,23	34,82	0,57	0,9	0,41	0,84	0,67	3,53	20,97	21,06	1,51	0,37	0,15
pH 3	1.09	0.33	0.12	0.12	0.04	0.53	4.59	5.61	35.52	0.45	0.63	0.32	0.59	0.56	1.88	24.64	20.04	1.43	0.17	1.25
pH 2.5	1,03	0,49	0,29	0,04	0,06	0,91	4,79	5,82	23,07	0,45	1,33	0,94	1,97	3,33	2,35	31,34	14,92	5,72	0,55	0,5

C10: 0: Capric acid.C12: 0: lauric acid.C14: 0: myristic acid. C14: 1: myristoleic acid. C15: 0 iso: Isopentadecylic acid. C15: 0 antieso: Antiesopentacyclic acid. C16: 1N7: palmitoleicacid. C16: 1n5: n-hexadecanoicacid. C16: 0: palmiticacid. C17: 0: margaricacid. C17: 0 iso: isomargaricacid. C17: 0 antieso: Antiesomargaricacid. C17: 1: heptadecenoic acid. C18: 0: stearic acid. C18: 1N9: oleic acid. C18: 1N7: vaccenic acid. C18: 2n6: linoleic acid. C18: 3n6: gamma-linolenic acid. C20: 0: arachidic acid. C20: 1N9:eicosenoic acid.

Membrane fatty acids analysis

The analysis of membrane fatty acids in normal and stressed *S. flexenri* cells were mentioned in Table III. For the normal strain, twenty fatty acids were identified including five main peaks such as palmitoleic acid (C16: 1N7), n-hexadecanoic acid (C16: 1n5), palmitic acid (C16:0), vaccenic acid (C18: 1N7) and linoleic acid (C18: 2n6). Their relative percentages were between 1.33% and 39.88%. Fifteen fatty acids were also detected at relatively low concentrations: C10: 0, C12: 0, C14: 0, C14: 1, C15: 0 iso, C15: 0 antieso, C17: 0, C17: 0 iso, C17 antieso, C17: 1, C18: 0, C18: 1N9, C18: 3n6, C20: 1N9 and C20: 0. Data analysis proved that the Fatty Acid composition was significantly ($P < 0.05$) influenced by all stresses applied in our study. The FA ratio (unsaturated to saturated) present in membrane was shown in Figure 3. These proportions proved the effects of these treatments on the unsaturation level of the Fatty Acid. Our results showed a significant decrease in the SFA proportion in most of the treatment except for pH 2.5 where an increase is noticed. In addition, the UFA-to-SFA ratio (Figure 4) was significantly decreased compared to normal cells with pH 2.5.

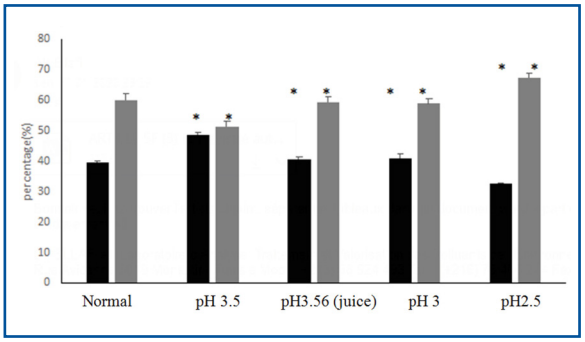

Figure 3. *Percentage of saturated fatty acids (■) and unsaturated fatty acids (■) composition (% of total FA) of Shigella flexneri under stressed conditions (*: P<0.05).*

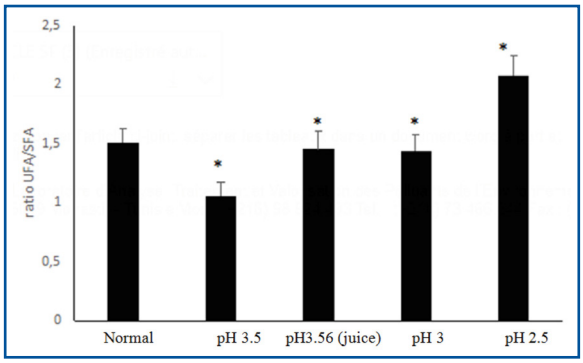

Figure 4. *Unsaturated-to-saturated ratio of* Shigella flexneri *CECT4804 under stressed conditions (*: P<0.05).*

Pathological Symptoms

At 5 × 10^8 CFU, rats had significant weight loss after 3 days infection, and died at the 7th day. Indeed, rats had severe diarrhea after 2 hours and the symptoms peaked 6 hours and continued until 12 hours as scored by fecal pathology after orally *Shigella* infection. Moreover, the symptoms of diarrhea and weight loss are aggravated, when the rats received stressful bacteria.

Histopathological findings

To further investigate whether oral challenge with *S. flexneri* (CECT 4804) can induce inflammatory symptoms in the large intestine, we initially examined morphological changes seven days post-infection. The control group exhibited normal intestinal tissue and mucosal structure with an intact epithelium (Figure 5A1). In contrast, tissues from rats treated with *Shigella* displayed cell death in the crypt and villi, along with a disrupted surface epithelium and inflammatory cellular infiltrate in the villous lamina propria of the intestinal mucosa (Figure 5A3). Histological sections of rat intestines subjected to stressed strains followed by *S. flexneri* (CECT 4804) infection revealed severely affected intestinal tissues compared to the treated rat group (Figure 5A7). The severity scores for intestinal lesions indicated a significant increase in histological scores in the diarrhea group compared to the control group. When compared to the normal group or those administered with broth, the results showed epithelial shedding, cell death in the crypt and villi, and infiltration of inflammatory cells in the rat intestine following *Shigella* infection.Moreover, histological sections of rat intestines after *S. flexneri* (CECT 4804) infection revealed distinct intestinal lesions compared to uninfected rats, with these lesions being more severe in stressed strains than in normal strains (Figure 5).

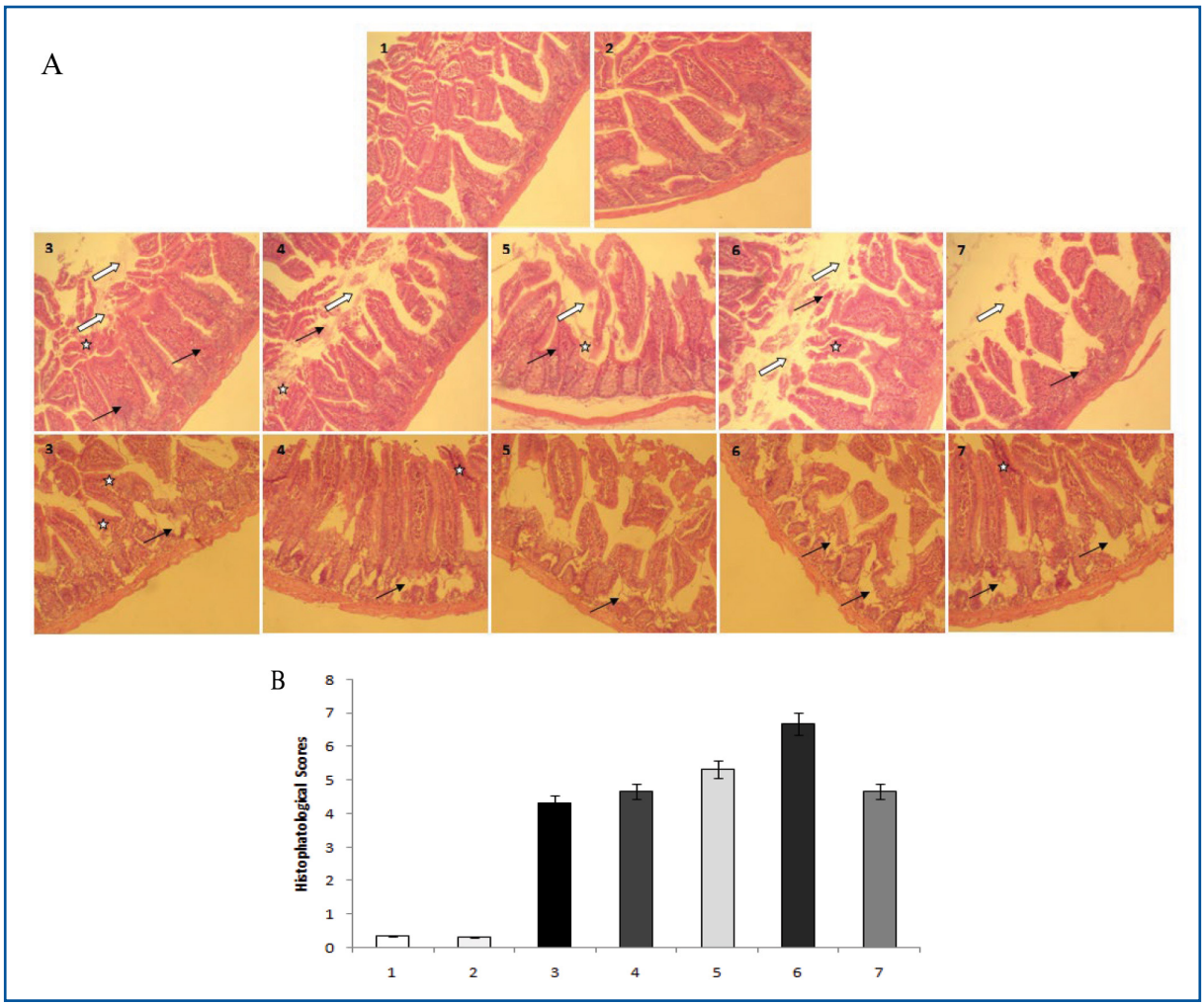

Figure 5: *(A) Histological analysis of rat intestinal tissues, and (B) Pathological scores of the intestine, indicating the severity of pathological intestine lesions. Rat intestinal tissues were fixed, embedded in paraffin and stained with hematoxylin and eosin. (1) Normal intestinal tissue and (2) Broth LB treated intestinal tissue, demonstrating a normal mucosal structure and intact epithelium. (3)* Shigella flexneri *CECT4804 infected intestinal tissue, demonstrating cell death in the crypt and villi (black arrow) and a disrupted surface epithelium (empty arrow) and inflammatory cellular infiltrate (star). (4) Rats were administered bacteria following pH stress (pH=3.5), (5) Rats were administered bacteria following pH stress (pH=3), (6) Rats were administered bacteria following pH stress (pH=2.5), (7) Rats were administered bacteria added with juice (pH=3.56); demonstrating severely infected intestinal tissue than group 3.*

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Discussion

The occurrence and persistence of human pathogen like *Shigella spp.* in many hostile environments, especially in wide variety of food, is increasingly being recognized as a significant threat to human health. The stress response of *S. flexneri* is complex, robust, and versatile. Bacterial stress adaptation of *Shigella* and the potential for stress-associated enhanced virulence need to be addressed in more detail to prevent potential risk of disease. An increased understanding of mechanisms and regulation of the stress adaptation will provide information for pathogenic control, increase effective design of novel control methods. The findings of this study illustrate, clearly, that bacterial cells of *S. flexneri* (CECT 4804) were able to survive at different pH values (7, 2.5, 3, 3.56 and 3.5) at 37°C. The obtained results for the different microcosms showed a decrease in the metabolic activity reaching a low level of viability after 48 hours. In addition, a proportional decrease was remarked in the viability related to the incubation time and the pH decrease. Also, the decrease in the *S. flexeneri* (CECT 4804) cells number especially at pH 2.5 and significant observed OD proving its resistance in this stressful environment are noticed. This result was confirmed by Abdul-aziz *et al*. 2015 whose mentioned that a pH 3 is lethal for the in-adapted strains. In accordance with our study, several results confirm the ability of *shigella flexneri* (CECT 4804) to survive in an extremely acid environment (pH=2.5) (Story *et al.* 2012; Zaika, 2001). The *S. flexneri* (CECT4804) persistence under extremely stressful conditions can be explained by the expression of certain genes when exposed to low pH values (Chan and Blaschek, 2005; Yang *et al.* 2015) as well as the synthesis or repression of such proteins (Abdul-aziz *et al.* 2015). Generally, gram-negative bacteria produces an outer membrane vesicles as well as a regulated process under different conditions (Baumgarten *et al.* 2012; Chen *et al.* 2014).

The bacterial stress response system is always related to expression of virulence factors, such as biofilm production which is strongly involved in adhesion. Indeed, slime production plays an important role in the infections of humans and antibiotic resistance of different microorganisms (Fux *et al.* 2005; Kouidhi *et al.* 2011; Pompilio *et al.* 2010). In this research, *S. flexneri* (CECT 4804) can produce biofilm under acid stress. In fact, this biofilm production may confer a resistance against the applied extreme acidity (Coughlan *et al.* 2016). Similar results have been found by Nickerson *et al.* (2017) who proved that the exposure of *shigella* to bile salt induces biofilm production and its adhesion to biotic surface. Different studies on other stressed bacteria show the same results. Thus, Baumgarten *et al.* 2012 show that for *Pseudomonas putida* in different stress conditions, the release of membrane vesicles (MV) as stress response leading to biofilm formation. In addition to the fact that thermal stress *Salmonella* use biofilm and resistance mechanisms (Villa-Rojas *et al.* 2017). Whereas, Kang *et al.* (2018) demonstrated that the Gallic acid had an inhibition effect on the biofilm formation.

In the same context, to cause the surface adhesion and the biofilm formation, several changes affect the bacterial membrane. The most important change affect the hydrophobicity capacity which plays a key role in the formation of biofilms and adhesion to biotic and abiotic surfaces (Krasowska and Sigler, 2014). In the present study, an increase in the hydrophobicity of stressed strains was registered. In a previous study, Xu *et al*. (2016) established a clear positive relationship between increased surface hydrophobicity and adhesiveness of *S. flexneri* according to Baumgarten *et al.* (2012) stress response leads to an increased hydrophobicity and consequently inducing biofilm formation.

The main function of the outer membrane of bacteria is to protect the bacterium from the external environment especially from toxic compounds such as antibiotics. It is also a boundary of the compounds transit exchanges between the intracellular and the extracellular mediums. In this study, changes in the fatty acids' membrane composition due to the acidity were showed. These changes modify the membrane fluidity. In fact, the results proved that acid stress causes fluctuations in lipid membrane composition and changes in UFA/SFA ratio which depend on the fluidity of the membrane. Indeed, cells regulate their lipid composition in order to reach a degree of fluidity suitable for life (Teixeira *et al.* 2002). On the one hand, changes in lipid composition in *Pseudomonas aeruginosa* occurred during the transition from the end of the exponential phase to the stationary phase Costerton, (1999). This is an obvious strategy used by cells to reduce membrane fluidity to save energy. On the other hand, previous study has shown that the surface properties of *Listeria innocua* cells, including the hydrophobicity of the cell and the fluidity of the membrane affected by the fatty acid composition, can be modified by exposure to various environmental stresses, including acid, heat stress and nutritional deficiency (Moorman *et al*. 2008). According to several studies, a membrane adaptation depends on an increase or a decrease in the unsaturated to saturated fatty acids ratio (UFA/SFA) and membrane fluidity is approved. They all depend on the stress applied type, cell physiological state and the growth phase (Alvarez-ordóñez *et al.* 2008; Chen and Gänzle, 2016; Teixeira *et al.* 2002).

Shigella spp is an invasive bacteria that uses a complicated process of pathogenicity implicating

different mechanisms on the basolateral side of epithelial cells, to cause infection and intestinal colonization (Wassef *et al.* 1989). The major mechanisms followed during shigellosis are invasion and inflammatory responses, Type III secreted effectors proteins that modulate inflammation and alterations in ion transport and barrier function (Hodges and Gill, 2010). In the present study, rats' infection with normal and stressed *S. flexneri* CECT 4804*,* induces various intestinal lesions. However, stressed strain at different pH values was more virulent causing additional intestinal destruction than normal one, especially at the lowest pH values. These results may be attributed to the *Shigella* pre-adaptation to the acidic medium, which was assured by different resistance mechanisms increasing its pathogenicity. According to Cornelis (2006) and Yang *et al.* (2014), the Type III Secretion System (T3SS) is the main responsible for diarrhea through virulent protein that disturbs the host defense system and facilitates infection. Moreover, to cause infection *Shigella* produces some enterotoxins, which have the ability to induce secretion of fluid in the intestine, thus causing a serious phenomenon of watery diarrhea (Niyogi, 2005; Niyogi *et al.* 2004; Sousa *et al.* 2013).

Conclusions

In conclusion, this study proves that acid stress influences the biofilm production and the surface hydrophobicity of *S. flexneri* (CECT4804). Also, it modifies the composition of the membrane fatty acids. This has an essential role in the survival of these bacteria under stressful conditions. The results of this study confirmed that stress enhances virulence by increasing host cell adhesion. These findings are very important in terms of explaining the pathogenesis of this bacterium when administered to rats. In fact, the intestine histological study of infected rats showed that stressed strain caused more intestinal lesion than normal one.

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