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Full Length Research Paper

Aeromonas hydrophila disturbs water and electrolyte transport in *Mugil cephalus* L. intestine

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Fish diseases create a menace to aquaculture farms. They provoke disastrous economic losses and sanitary risks for the consumer. The present study aims to investigate the effect of the bacteria, *Aeromonas hydrophila* on water and electrolyte (Na^+, K^+, Cl^-, HCO_3^-) flux of *Mugil cephalus* (L, 1758) intestine. Anterior, middle and distal gut segments of *M. cephalus* (L) intestine were used in an *in vitro* model; Everted Gut Sac (EGS). The sacs were exposed to bacteria suspension (10^8 cells/ ml) at 25°C for 2 h. Our results showed a significant reduction of water absorption at the anterior and the mid intestine ($P< 0.05$), and a significant increase of K^+ secretion only at the anterior intestine ($P < 0.01$). However, HCO_3^- secretion increase was significant at the anterior and the mid intestine ($P < 0.05$). Paradoxically, an increased absorption of Na^+, and Cl^- was recorded at the mid ($P < 0.01$) and at the distal gut segments ($P < 0.05$). Histological studies were assessed by light microscopy. EGS exposed to Ringer solution (12%, pH 8.5) revealed the presence of intact intestinal tract. However, infected EGS showed intestinal damages characterized by epithelium lesions, detachment of degenerate enterocytes with voluminous and spherical shape, disappearance of enterocyte brush border and lesions at cellular junction. It can be concluded that *A. hydrophila* resulted in a disturbance of hydroelectrolytic flux and alterations of *M. cephalus* intestinal tract. The most serious damage was noted at the anterior segment.

Key words: Everted Gut Sac, *Mugil cephalus*, *Aeromonas hy*drophila, water flux, electrolyte flux.

INTRODUCTION

Bacteria infection of fish constitutes a huge menace for aquaculture farming, leading to disastrous economic loss and health risks for the consumer (Lau et al., 2007). Numerous bacterial species have been recognised as pathogenic, causing fish diseases. Several studies have shown that Aeromonas affects both fish and sea fruit (Paniagua et al., 1990) and causes different diseases. For instance, Furunculosis is caused by *Aeromonas salmonicida*. Haemorrhagic septicaemia and ulcer were as well attributed to *Aeromonas hydrophila* (Dierckens et al., 1998).

Fish gastrointestinal tract is one of the major infection tracts. It presents a favourable medium for bacteria multiplication (Robertson et al., 2000; Ringø et al., 2002). Bacteria translocation across intestinal epithelium is the passage of bacteria from the intestinal tract into the enterocytes. It is an important step of bacteria invasion. It is at the origin of morphologic alteration by the production of virulent factors such as endotoxin, cytotoxin and hae-

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molysins (Chopra et al., 2000; Jutfelt et al., 2006). The damaging effects have been described for *Salmo solar* L (Ringø et al., 2004).

Mugil cephalus (L) (flat head) is a euryhaline species present on the Tunisian coasts for a long period of the year. It presents a huge economic interest and it is equally appreciated by the consumer.

The major aim of our study was to explore, using an *in vitro* model [Everted Gut Sac (EGS)], the relationship between the infection provoked by *Aeromonas hydrophila* of *M. cephalus* intestine, the damages recorded at the intestinal tract and the disturbance of water and electrolyte transfer.

MATERIALS AND METHOD

Bacteria strain

The strain used was *Aeromonas hydrophila*. It was isolated from infected *Dicentrarchus labrax* (Linnaeus, 1748) liver and spleen. It was kindly donated by the Laboratory of Analysis, Chemical Control and Microbiological pollutants of Environment (Faculty of Pharmacy Monastir, Tunisia). The bacterium was stored at - 80°C in tubes containing glycerol (20%), and then inoculated into nutritive Marine broth for 24 h. This culture was inoculated into nutritive gelose Marine broth for 24 h. The suspension obtained was adjusted to a transmission of 610 nm by adding freshly prepared Ringer solution (12%) in g /l (NaCl, 12; KCl, 0.35; HCO_3 Na, 0.28 and $CaCl_2$, 0.42 g). pH was adjusted to 8.5 and the suspension bacteria concentration was equal to 10^8 cells/ml. All chemical products were regent grade and obtained from Prolabo (France).

Fish

M. cephalus (L) (flat head) (Linnaeus, 1758) were obtained from Hergla lake and brought (in a specifically adapted aquarium with a ventilation system) to the National Sea Institute of Sciences and Technology of Monastir, Tunisia. The Fish were held in a tank with a flow aerate using renewed sea water for at least 2 weeks prior to the experiments. The temperature was maintained between 18 and 22°C and the fish were fed on a standard diet composed of algae.

Treatment of animals

The Fish were anesthetized with 0.1% 3-aminobenzoic acid ethyl ester. The intestines were immediately removed, stripped of adhering tissue and cleaned carefully with Ringer solution (12%, pH 8.5). Anterior, medium and distal gut intestine were divided into different segments (medium length is of 7 cm). EGS were prepared as previously described (Khemiss et al., 2006). The EGS were held up in an incubatory medium containing Ringer solution (12%, pH 8.5) in the presence and the absence of bacteria suspension. The EGS were filled with 400 µl of Ringer solution (12%, pH 8.5), maintained at 25°C and bubbled with gas (O_2/CO_2) 95/5. Incubation time was 2 h.

Determination of water and electrolytes flux

Water flux was determined in the presence and in the absence of *A. hydrophila*. The results were expressed in mg of water/g of intestine/h (Charpin et al., 1992; Khemiss et al., 2005). Electrolytes flux of Na^+ and K^+ were determined by photometry (Flame photometer BT634, Biotecnica instrument, Italy) (Charpin et al., 1992; Marzougui et al., 2006). Electrolytes flux of Cl^- was determined according to the Shales and Shales method (Shales and Shales, 1941). Electrolytes flux of HCO_3^- was determined by an automat (Ultra M Norme Biomedical, USA). The values were expressed in µmol/g of intestine/h.

Histological study

Different tissue samples (control and infected EGS) were fixed by immersion in formol and then dehydrated and embedded in paraffin. Serial 8 µm transversal sections were deparaffinized and stained with hematoxylin and eosin. Slides were then dehydrated in graded series of ethanol solutions, immersed in toluene and mounted with Canada Baume before observation by Light Microscopy (Martoja and Martoja, 1964). Tissue sections were observed and photographed on an axioskop microscope (Zeiss).

Statistical study

Statistics were computed with the PSS for windows. All values are expressed as the mean ± SEM. Statistical significance of results was analyzed by student "t" test. The results were considered significant for $P < 0.05$.

RESULTS

Water flux

During the control conditions, water absorption was noted at the anterior, mid and distal gut segments. The flux was respectively 190 ± 16.96; 165 ± 7.29 and 103 ± 3.03 mg of water/g of fresh intestine/h (Figure 1). The addition of *A. hydrophila* (10^8 cells/ml) in the incubatory medium (Figure 1) produced a decrease of absorption at the anterior and at the mid gut segment ($P < 0.05$). At the distal gut segment the presence of the bacteria caused no significant change in water flux.

Electrolyte flux

In the absence of bacteria suspension and following 2 h of incubation time, a Na^+ absorption was noted at the different segments of *M. cephalus* intestine (Table 1). The addition of bacteria suspension (10^8 cells/ml) to the incubation medium led to a significant increase of Na^+ absorption at the mid ($P < 0.01$) and at the distal gut segments ($P < 0.05$). However, at the anterior segment, absorption variation was not significant. The results recorded for the Chloride uptake equally showed an absorption of 14.92 ± 0.75; 2.3 ± 0.51; 12.21 ± 0.19 µmol/g of fresh intestine/h respectively at the anterior, mid and distal gut segments (Table 1). The presence of

Table 1. Variation of electrolyte flux in *M. cephalus* (L) intestine in the presence and the absence of *A. hydrophila.*

Part of intestine	Na^+		Cl^-		K^+		HCO_3^-	
	Control	Infected	Control	Infected	Control	Infected	Control	Infected
Anterior (n=9)	14.08±0.34	9.16±1.07 NS	14.92±0.75	25.58±1.5*	-0.33±0.04	-1.16±0.14**	-1.7±0.1	-2.05±0.23*
Mid gut (n=10)	3.23±0.61	14.76±1.11**	2.3±0.51	19.6±1.21**	-1.03±0.04	-1.12±0.1NS	-1.72±0.08	-2.03±0.04*
Distal gut (n=10)	10.56±0.51	18.08±1.19*	12.21±0.19	19.08±1.0*	-1.44±0.07	-1.36±0.2NS	-1.93±0.07	-2.25±0.32NS

Flux of Na^+, Cl^-, K^+ and HCO_3^- were determined from the liquid inside the EGS and expressed in µmol/g of fresh intestine/h.
** $p < 0.01$ Difference highly significant between control and infected intestinal segments; and *$p < 0.05$ Difference significant between control and infected intestinal segments. Control intestinal segments are EGS incubated in Ringer solution (12‰, pH 8.5) for 2 h at 25°C. Infected intestinal segments are EGS incubated in bacteria suspension of *A. hydrophila* (10^8 cells/ml) for 2 h at 25°C.
n = Number of segment used for each experience.

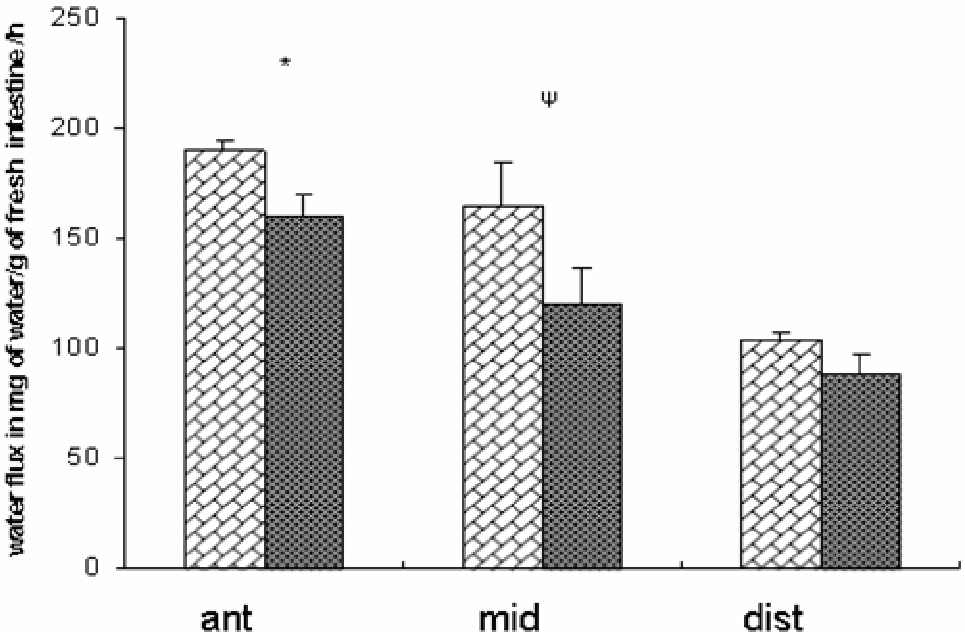

Figure 1. Effect of *A. hydrophila* on the variation of water flux on the different segments of *M. cephalus* (L) intestine anterior (ant), medium (mid) and distal gut segment (dist) after 2 h of incubation time. Water flux is expressed in mg of water/g of fresh intestine/h. The addition of *A.hydrophila* suspension (10^8 Cells/ml) in the incubation medium for 2 h induces a significant (*) decrease of water flux at the anterior segment ($p < 0.05$) and a significant (ψ) decrease of water flux at the mid segment ($p < 0.05$). But at the distal gut segment the difference between infected and control sample is not significant. Control samples were treated with a Ringer solution12% and infected samples were incubated in a bacteria suspension of *A. hydrophila* (10^8 cells/ml) for 2 h.

A. hydrophila (10^8 cells/ml) in the incubation medium significantly enhanced the Chloride absorption at the mid gut segment ($P < 0.01$). At the anterior and the distal gut segment, the increase was also significant ($P < 0.05$). For Potassium, a secretion at the different segments studied was noted. *A. hydrophila* produced a large secretion increase (Table 1) at the anterior intestine ($P < 0.01$). Bicarbonates were equally secreted at the three intestinal segments. The exposure of the EGS to *A. hydrophila* significantly increased this transfer at the anterior and

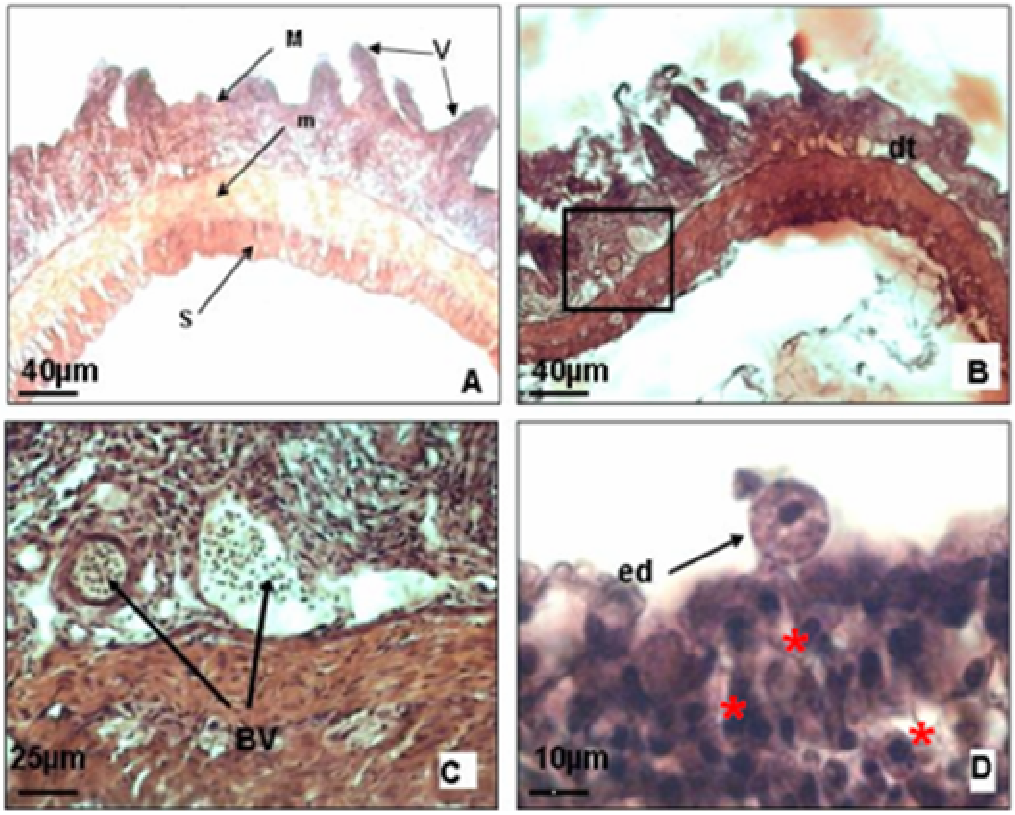

Figure 2. Light micrograph of the histological views for anterior intestine of *M. cephalus* (L) after 2 h of incubation. **A.** (x 5) .Bar: 40 µm . Control with Ringer solution 12% pH 8.5. Coloration Hematoxylin Eosin (HE). The figure shows 2A normal intestine with three layers M: mucosae, m muscularis and S: serosa. We note also the presence of: villosities. V. **B.** (x5) Bar: 40 µm. Bacteria suspension of *A. hydrophila* (10^8 cells/ml). Coloration Hematoxylin Eosin (HE). Figure 2B showing dt, detachment of layers intestine; BV, blood vessel including heterogenous cell population. **C.** (x40) Bar: 25 µm. Coloration Hematoxylin Eosin (HE). The figure 2C shows BV, blood vessel including heterogenous cell population. **D.** (x100) Bar: 10 µm. Coloration Hematoxylin Eosin (HE). The figure 2D shows ed, rounded and swollen entrocyte and enlarged intercellular spaces*.

mid gut segments ($P < 0.05$), while this secretion was not significant at the distal gut segment (Table 1).

Histological study

Histological slides of *M. cephalus* intestine, observed by light microscopy in a Ringer solution (12 %, pH 8.5) at 25°C and after 2 h of incubation time showed normal structure of fish intestinal tract with three intestinal layers: serosa, muscularis and muscularis mucosae with high developed villosities into the lumen (Figure 2A, Figure 3A and Figure 4A). The presence of microvillosities (Figure 3C) and intact border brush were noted (Figure 4C). The exposure of the EGS to *A. hydrophila* for 2 h induced morphologic alterations observed at the anterior intestine:

(i) Epithelium lesion, detachment of intestinal layers and apparition of altered cells (Figure 2B).
(ii) Brush border disappearance with discontinued detachment of villosities and blood vessel including heterogeneous cell population (Figure 2C).
(iii) Enlarged intercellular spaces and degenerate enterocytes with voluminous and spherical Shape (Figure 2D).

At the mid and distal gut segments, the presence of disintegrate enterocyte and local disappearance of border brush were noted (Figure 3D and Figure 4D).

DISCUSSION

EGS is an *in vitro* model. It is a helpful technique permitting simultaneous evaluation of water and electrolytes flux (khemiss et al., 2005; Marzougui et al., 2006). The experimental model has been previously employed

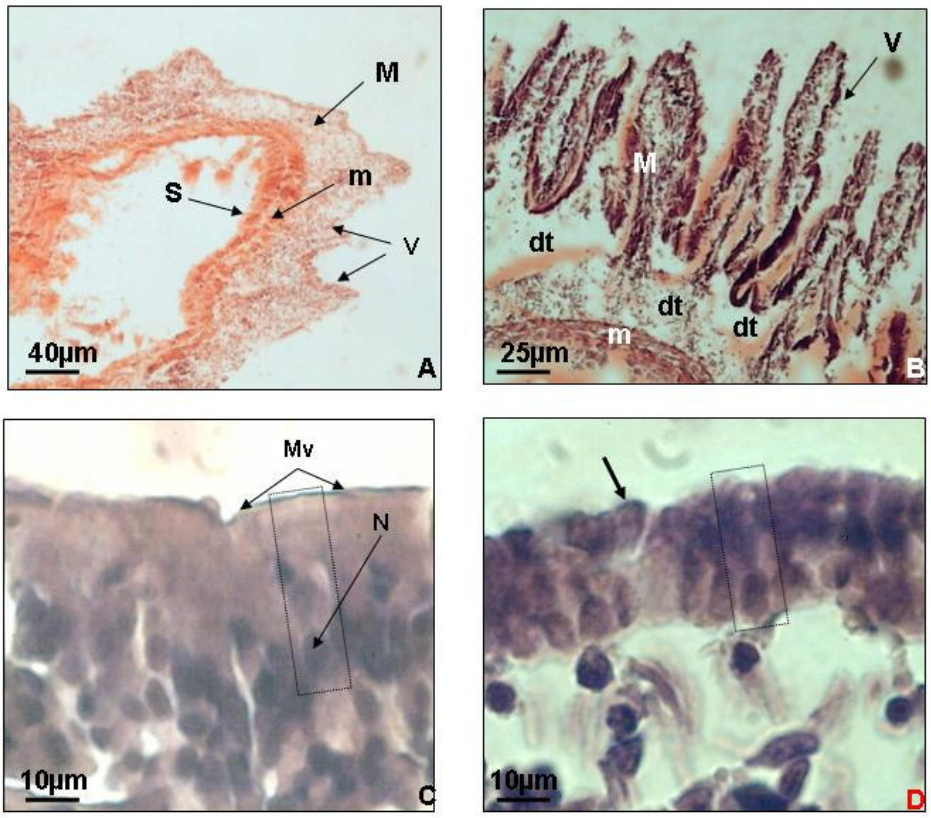

Figure 3. Light micrograph of the histological views for mid intestine of *M. cephalus* (L) after 2 h of incubation.
1. To Ringer solution 12‰.
Figure 3 A (x 5) Bar: 40 µm .Coloration Hematoxylin Eosin (HE). Figure 3A Shows normal intestine with three layers M: mucosae, m: muscularis; and S: serosa;
We note also the presence of: villosities. V
Figure 3C(x100) Bar: 10 µm .Coloration Hematoxylin Eosin (HE)
Figure 3C(x100): Mv: microvillosities; E: enterocytes, N: nucleous.
2. To bacteria suspension *of A. hydrophila* (10^8 cells/ml)
Figure 3B (x40) Bar: 25 µm .Coloration Hematoxylin Eosin (HE)
Figure 3 B detachment (dt) of mucosae (M) and muscularis (m),
We note also the presence of preserved villosities. V
Figure 3D (x100) Bar: 10 µm .Coloration Hematoxylin Eosin (HE)
Figure 3D: desintgrates enterocytes E and local diseaperance of border brush

(Moshtaghie and Taher, 1993; Marzougui et al., 2006). The validity of this model by a histological study was explored in a previous data (Khemiss et al., 2006). This model was also extensively used exploring fish intestine (Smith et al., 1975; Tritar et al., 1983; Hogstrand et al., 2002).

The data presented in this study used *A. hydrophila*, a pathogenic fish bacterium (Paniagua et al., 1990). Fish bacteriosis is one of the continuous economic problems for aquaculture farm and it is responsible for sanitary risks threatening the consumer (Lau et al., 2007). Fish gastrointestinal tract constitutes one of the major infection routes like the skin and the gills. It presents a favourable site for bacteria proliferation (Robertson et al., 2000). This proliferation inside the gastro-intestinal tract may induce a disturbance of the hydro-electrolyte flux.

In our experimental condition, water absorption occurred at the different intestinal segments of *M. cephalus*. This transfer was carried from the mucosal side to the serosal side. Ando et al. (1986) showed the same way of water transfer. Similar results were recorded in rainbow trout (Bensahla et al., 1974). However, water absorption may be different from one fish species to another. The flux was constantly the same along the intestinal tract in

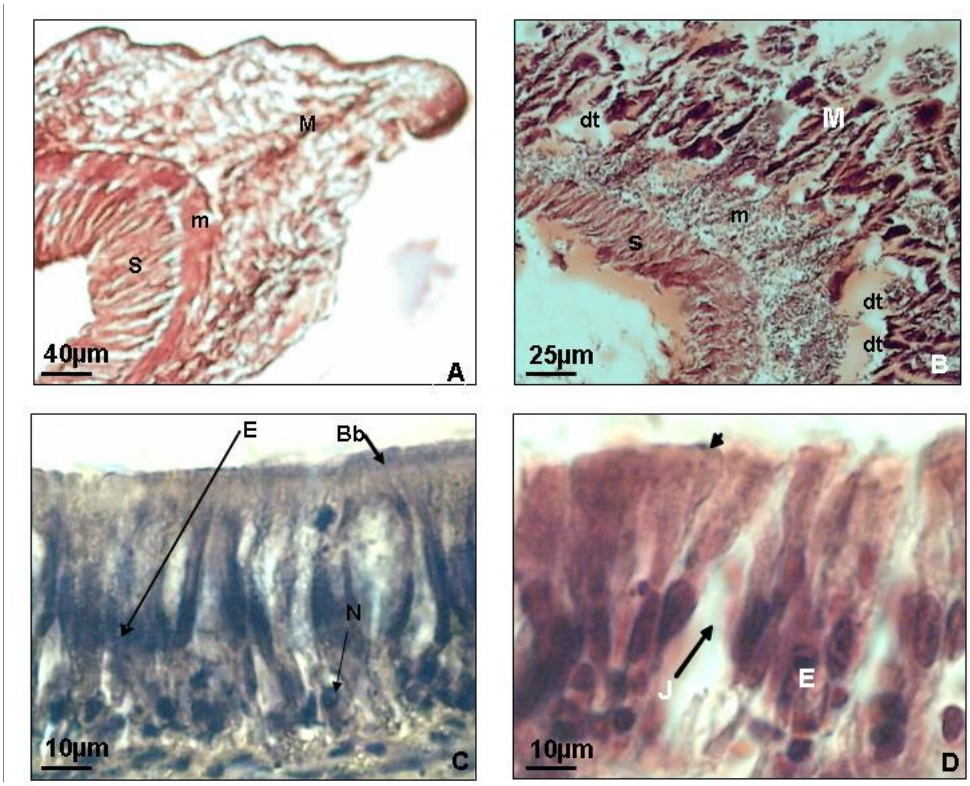

Figure 4. Light micrograph of the histological view for distal gut intestine of *M. cephalus* (L) after 2 h of incubation
1. to Ringer solution 12‰
Figure 4 A (x 5) Bar: 40 µm .Coloration Hematoxylin Eosin (HE)
Figure 4 A(x5): shows normal intestine with three layers Mucousae : M, muscularis m and serosa: S.
We note also the presence of villosities.:V
Figure 4 C (x100) Bar: 10 µm .Coloration Hematoxylin Eosin (HE)
Figure 4C(x100): showing enterocyte: E and nucleous: N
2. to bacteria suspension of *A. hydrophila* (10^8 cells/ml)
Figure 4B(x40) Bar: 25 µm .Coloration Hematoxylin Eosin (HE)
Figure 4 B(x5) showing detachment of intestine layers and some preserved villosities
Figure 4D(x100) Bar: 10 µm .Coloration Hematoxylin Eosin (HE)
Figure 4D(x100): showing enterocytes detachment (E), and alteration junctions (J) ⟶
presence of discontinued border brush ➔

Carrassinus auratus (Smith, 1964) and was highly increased at the mid gut intestine for *Anguilla japonica* (Ando and Nagashima, 1996). However, Aoki et al. (2003) found that the large water absorption occurred at the posterior intestine of *Anguilla japonica*.

Concerning electrolyte transfer, our results showed a reabsorption of sodium and chloride. We noted that chloride flux exceed sodium flux at the different intestinal segments. This result may assure chloride equilibrium and facilitate bicarbonate secretion (Scott et al., 2006). It has been reported that absorption of Na^+ and Cl^- seems to be a coupled process (Burgess et al., 2000). This phenomenon is insured by an apical cotransport Na^+ - Cl^- (Movileanu et al., 1998; Marshall et al., 2002; Scott et al., 2006). The presence of an apical $Na^+/K^+/2Cl^-$ cotran-sport permitted sodium and chloride absorption (Ando et al., 2003). It is at the origin of electrochemical gradient inducing water transfer from mucosal to serosal side (Ando et al., 1986). The energy is supplied by Na^+/K^+ ATPase located in basolateral membrane (Trischitta et al., 2004; Scott et al., 2006).

For potassium and bicarbonate, a secretion was noted at the different segments of *M. cephalus* (L) intestine. Our results agree with those of Wilson et al. (2002). They reported variable bicarbonate secretion at the intestinal teleosteen fish. The phenomenon of secretion involved

both:

(i) A basolateral Cl^-/ HCO_3^- exchanger in correlation with electrochemical gradient for Cl^- (Dixon and Loretz, 1986).
(ii) An apical Cl^-/ HCO_3^- exchanger in relation to an electrochemical gradient of HCO_3^- (Grosell et al., 2004).

The equilibrium of fluid movements through the intestinal tract results from the absorption and secretion phenomena. Aquaporins are channel protein implied in this fluid transport (Ma and Verkman, 1999). Several types of aquaporins with a variable distribution and localization have been shown. These variations in the distribution and the localization were detected immunohisto-chemically (Lignot et al., 2002). These variations may explain the difference of water flux observed in our data for control samples.

The addition of the bacteria in the incubation medium caused a decrease in water absorption at the anterior, mid and distal gut segments of *M. cephalus*. Similar results were noted at *Sparus aurata* intestine exposed to *Vibrio vulnificus* (data not shown). However, in mammal intestine, bacteria effects were different. Epple et al. (2004) noted a decrease of water absorption in the presence of *A. hydrophila*, while Marzougui et al. (2006) using *Salmonella typhimurium*, recorded a water increase secretion. Interestingly, our results showed an increase reabsorption of sodium and chloride in the presence of *A. hydrophila*. Chloride flux always exceeded sodium flux. This result allowed to explain the secretion increase of bicarbonate in the presence of *A. hydrophila* (Scott et al., 2006). Our results agree with previous studies on fish intestine exposed to toxin which revealed increase of chloride flux (Loretz 1983; O'Grady 1989). It seems that this phenomenon is a specificity of teleosteen fish (Lahlou and Avella, 1993). The presence of *A. hydrophila* in the incubatory medium stimulated the potassium secretion. This result may be related to the activity of $3Na^+$, $2K^+$ pump in the lateral membrane intestine (Scott et al., 2006).

Histological sections of *M. cephalus* intestine were observed by light microscope. Three layers were identified; serosa, muscularis and muscularis mucosae with villosities into the lumen. Histological studies of intestine after 2 h of incubation time in a Ringer solution (12‰, pH 8.5) at 25°C showed that the intestine remained intact morphologically with highly developed villosities (Figures 2A, 3A and 4A). In our experimental conditions, *M. cephalus* intestine preserved its viability and its functionality. The cellular damage was recorded after the *M. cephalus* intestine was exposed to *A. hydrophila*. However, the most severe lesions were noted at the anterior segment. The damage involved epithelium lesion (Figures 2B and 2C), degenerate enterocyte with voluminous and spherical Shape (Figure 2D).

Previous studies have shown that pathogenic bacterial strain provoked damage to fish intestine. Our results agree with those of Ringø et al. (2003) whose results were recorded at the anterior intestine of *Anarhichas minor* infected by *V. anguillarum*. Ringø et al. (2004) also confirmed the results already shown at *Salmo salar* (L) intestine infected by *Aeromonas salmonicida* ssp. *salmonicida*. Those histological changes were also noted at *S. salar* (L) intestine exposed to pathogenic bacterial strains (Bakken, 2002; Ringø et al., 2004, 2007). The results presented in Figure 2B show a detachment between muscularis and mucosa layers. The presence of cells grouped in blood vessels was noted. This type of cells may present infected and swollen enterocyte as a result to an inflammatory reaction. These observations were signalled in mammal caecum by Kunkel and Rosenthal (1986).

Morphological modifications were also noted at mid and distal gut segments. They were less severe. Brush border alteration was discontinued. The presence of preserved villosities and enterocytes were noted in Figures 3B, 3D, 4B and 4D. The presence of endogenous intestinal flora in teleost fish intestine was variable from one segment to another. The abundance of this flora was located at the distal gut segment (Moran et al., 2005). This difference of flora abundance may explain the different degrees of alteration noted at the three intestinal segments of *M. cephalus* intestine. Therefore, the exploitation of histological slides of *M. cephalus* intestine indicates that the different part of fish intestine present variable sensibilities against pathogenic factors (Ringø et al., 2003). Based on our findings, we propose that *A. hydrophila* would induce damage to *M. cephalus* intestine with a specific action at the anterior segment.

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