SYSTEMATICS, MORPHOLOGY AND PHYSIOLOGY

Occurrence of Apocrine Secretion in the Larval Gut Epithelial Cells of *Aedes aegypti* L., *Anopheles albitarsis* Lynch-Arribálzaga and *Culex quinquefasciatus* Say (Diptera: Culicidae): a Defense Strategy Against Infection by *Bacillus sphaericus* Neide?

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Ocorrência de Secreção Apócrina nas Células Epiteliais do Intestino de Larvas de *Aedes aegypti* L., *Anopheles albitarsis* Lynch-Arribálzaga e *Culex quinquefasciatus* Say (Diptera: Culicidae): um Mecanismo de Defesa contra Infecção Causada por *Bacillus sphaericus* Neide?

RESUMO - Observou-se hipertrofia das células do epitélio intestinal de *Aedes aegypti* (L.), *Anopheles albitarsis* (Lynch-Arribálzaga) e *Culex quinquefasciatus* (Say) devido ao aumento da atividade secretora intracelular como primeira reação às toxinas das linhagens 2362 e S1116 de *Bacillus sphaericus* (Neide). Após a hipertrofia epitelial, formaram-se vesículas na porção apical das células, as quais eram compostas de fragmentos de membrana plasmática contendo material de natureza desconhecida em seu interior, caracterizando um tipo de secreção apócrina. A via de contaminação das larvas dos mosquitos por essas bactérias é pelo intestino, através da alimentação. Dependendo da espécie de Culicidade e da linhagem bacteriana utilizada, a hipertrofia do epitélio ocorreu entre 5 a 15 min após a exposição das larvas ao meio contaminado. O segundo aspecto observado no processo de contaminação foi o aumento da atividade de secreção apócrina. As vesículas basófilas que se desprendiam das células permaneciam entre a membrana peritrófica e o lúmen intestinal. Observou-se atividade secretora tanto no grupo controle como no experimental, porém muito mais intensa no grupo experimental. Os Culicidae estudados apresentaram diferenças marcantes nas respostas às toxinas das bactérias utilizadas, sendo *C. quinquefasciatus* a espécie mais suscetível. As diferenças de reações das células em relação às toxinas produzidas pelas duas linhagens bacterianas são discutidas.

PALAVRAS-CHAVE: Mosquito, controle biológico, virulência

ABSTRACT - Cell hypertrophy was the first reaction of the gut epithelial cells of *Aedes aegypti* (L.), *Anopheles albitarsis* (Lynch-Arribálzaga) and *Culex quinquefasciatus* (Say) to the toxins of strains 2362 and S1116 of *Bacillus sphaericus*, as cells had an increase of intracellular secretory activity. Soon after the cell hypertrophy developed, vesicles were formed at the cell apical portion, which detached with the plasma membrane, characterizing a type of apocrine secretion like. The first pathway of contamination of the mosquito larvae by these bacteria is through the gut, by feeding. Depending on the species of Culicidae and on the bacterial strain used, the hypertrophy of the gut epithelium occurred between 5 and 15 min after exposure to the contaminated environment. The second aspect observed after hypertrophy was the increase in apocrine secretion. The basophilic vesicles that detached from the cells remained between the peritrophic membrane and the gut lumen, such vesicles were filled with material of unknown nature. The gut posterior region showed secretory activity in both control and treated larvae, being much more intense in bacteria-exposed larvae. There were remarkable differences in the epithelial cell reaction according to the toxins of the two bacterial strains, but *C. quinquefasciatus* was the most susceptible. Differences in the gut cell reactions to the toxins produced by the two bacterial strains are discussed.

KEY WORDS: Mosquito, biological control, virulence

Natural biological control of *Aedes* (Meigan), *Anopheles* (Meigan) and *Culex* (L.) mosquito larvae in Amazonia has become one of the most important alternative to prevent development of these mosquitoes, as they are vectors of many human diseases. Laboratory analyses of the bacterial virulence or toxicity have shown the potential of toxic strains of *Bacillus sphaericus* as a bacteriological agent for mosquito control (Kellen & Meyers 1964). Efforts to assess the toxicity of these strains, their viability for use in the field, as well as their possible effects on the target insect are important contributions to the implementation of strategies for mosquito control and for the prevention of possible effects to the associated, non-target entomofauna.

The mosquito larval digestive system is the first target of pathogenic bacteria, which penetrate the gut lumen during the larval feeding stage, causing many morphological injuries in the entire gut of the disease vector mosquito. The bacterial toxin causes several types of damage to gut epithelial cells, such as cytoplasm vacuolization, microvillus alteration, increase of the secretory cell activity and extrusion of the cell vesicles with intracytoplasmic material inside, and finally, cell death (Karch & Coz 1983, Percy & Fast 1983, Cavados *et al* 2004).

In some aquatic dipterous the process of infection starts 90 min after bacterial exposure, when the gut microvillar cells disappear, leaving only a flat, apical plasma membrane. At 120 min after infection, the epithelial cells are totally damaged (Habib 1983). Therefore, histopathological analyses can act as an important tool in providing essential information on the toxicological capacity of a bacteria of interest for a defined period of time.

The focus of the present study was to contribute to the analysis of the histopathology of *Bacillus* infections to attempt to understand the morphological changes of larval intoxication in the gut of some Culicidae species.

Material and Methods

Collection of the culicids. Eggs of *Aedes aegypti* (L.) were collected at domiciles in the region of Manaus (AM) by using slot-bearing ovitraps, taken to the lab, allowed to dry for 24h, and then placed in enameled containers holding water and liquid food for immature development (4th instars). *Anopheles albitarsis* (Lynch-Arribálzaga) were collected in the periphery of Manaus and at Cacau Pereira in the Township of Iranduba (state of Amazonas, AM), between 6 p.m. and 9 p.m. Mosquito females were captured in cattle pens with the aid of entomological traps and stored in paraffin-coated vials, and maintained captive so they would lay their eggs and provide immatures (3rd and 4thinstars). *Culex quinquefasciatus* (Say) were collected in the region of Manaus from premise to premise, using barge-shaped aluminum shells for egg collection. Eggs were then placed in enameled containers with water and liquid food for specimens rearing and analysis (4th instars).

Sample preparation. Fifty 4th instars of each species, *A. aegypti*, *A. albitarsis* and *C. quinquefasciatus*, as well as 3rd instars of *A. Albitarsis*, were placed separately in 100 ml cups containing 50 ml deionized water, 1 ml food, and 1 ml (0.2 ppm) of a solution of *Bacillus sphaericus*. Readings were taken at 5, 15, 30 and 45 min, and 1, 2, 3, 4, 5, 6, 12, 24, 48 and 72h after bacterial exposure. The bioassays were carried out three times at 26 ± 2ºC and 80% relative humidity.

Larvae were collected each time with the aid of a medicine dropper and placed in 0.5 ml and 1.5 ml tubes containing a fixative solution (4% paraformaldehyde in cacodylate buffer, 0.1M, pH 7.2), and stored at room temperature until further processing.

Histological analysis. Samples were fixed in 4% paraformaldehyde in cacodylate buffer (0.1 M, pH 7.2) for at least 2h, dehydrated in graded ethanol (70% to 95%) before being embedded in historesin Leica® following the procedures to routine optical microscopy. Sections of 7 µm were obtained in microtome Leica®, stained with haematoxylin and eosin (HE), and observed with a Zeiss® optical microscope.

Results

Culicidae general larval digestive tract morphology. Analysis of the cell morphology of the digestive system of *A. aegypti*, *A. albitarsis* and *C. quinquefasciatus* indicated similarities in the morphological compartmentalization of the gut, which is divided into foregut, midgut and hindgut (Fig 1A). In the foregut, the gastric caecum is located in the thorax and comprises a loop in the digestive tract, resembling two symmetrical loops at the cephalic region, close to the oral cavity. It was possible to observe food in the gut lumen, which has an epithelium of columnar cells (Fig 1A,B). The gastric caeca cells did not contain well-developed microvilli (brush borders). Intercellular boundaries are not clearly defined, and cell nuclei are located in the basal region of the cells (Fig 1A,B). Usually, in both the control and experimental groups, acidophilus vesicles, which appear detached from the apical portion of gastric epithelium cells undergoing extrusion, are observed in the lumen of the gastric caeca (Fig 1A). Midgut cells are cubical, with well-developed microvilli and central nuclei generally containing de-condensed chromatin and a central nucleolus (Fig 1C). The cytoplasm of the cells at these gut portions is usually more intensely stained by eosin than that of the hindgut cells (Fig 1B-D). The foregut and midgut are located between abdominal segments IV and I. The hindgut is easily distinguished from the other gut portions by its clearly hypertrophied epithelium (Fig 1C). It usually begins between abdominal segments IV and V. The cytoplasm of the cells of the hindgut epithelium is very basophilic**,** being much more densely stained by haematoxylin than by eosin. Cells are columnar in shape, and the epithelium carries developed microvilli as well as nuclei with de-condensed chromatin, and is apparently more active, with evident nucleoli, and a sinuous, elongated shape (Fig 1D).

Gut cells are not in direct contact with the food, but are isolated by a cuticular acellular membrane called peritrophic membrane (Fig 1B-D), which compartmentalize the gut in ectoperitrophic and endoperitrophic space (Fig 1B-D).

Histopathology. Third instars of *A. albitarsis* exposed to the

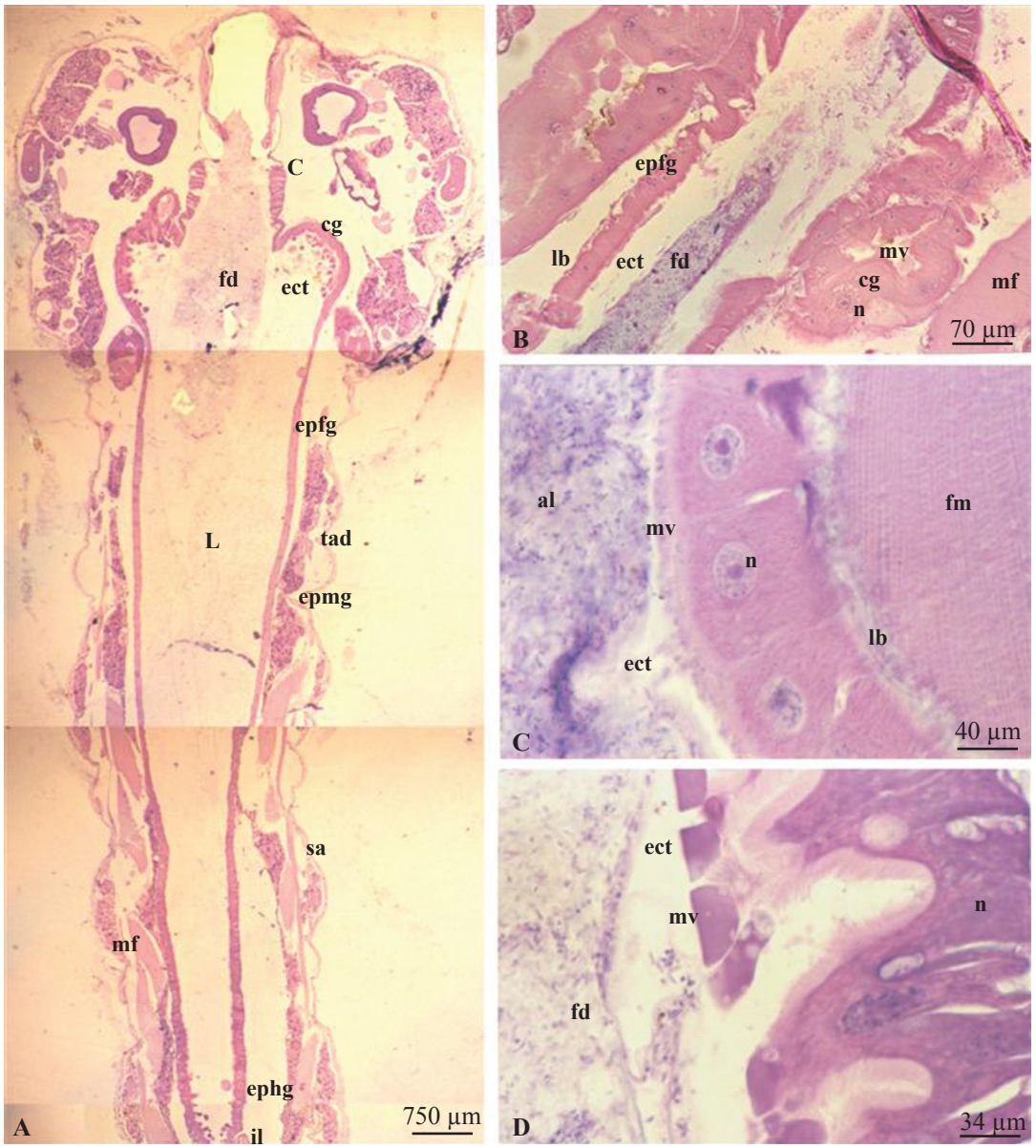

Fig 1 Histological sections of 4th instars of *Anopheles albitarsis* after exposure to bacilli 2362. (A) Fore-, mid- and hindgut after 6h. (B) Cells of the foregut after 6h. (C) Cells of the midgut after 10 min. (D) Cells of the hindgut after 10 min. lb, basal lamina; C, cardia; ect, ectoperitrophic space; epfg, epithelium of foregut; ephg, epithelium of hindgut; epmg, epithelium of midgut; fd, food in gut lumen; cg, gastric caecum; L, gut lumen; mf, muscle fibers; mv, microvilli; n: nucleus; tad, degenerated epithelium of the tegument.

S1116 bacterial strain showed epithelial cell hypertrophy (Fig 2A,B). After 4h of exposure, the gut was notably hypertrophied and showed active synthesis of some acidophilic secretion extruded from the cell by apocrine secretion (Fig 2B). After 6h, the midgut cells lost contact with their basal membrane and appeared totally disorganized and vacuolated (Fig 2C).

In 4th instars exposed to bacterial strain S1116, the cells became highly columnar and many more basophilic vesicles migrated to the ectoperitrophic space after 10 to 30 min of exposure; some of them could be observed detaching from the gut epithelial cells (Figs 3B,C,H). The increase in nuclear activity was evident from the de-condensed chromatin and very large nucleolus (Figs 1B,C). The cells were closely attached to the well-developed basal lamina, which followed the sinuous labyrinth of the plasma membrane of the basal portion of the epithelial cells, constituting another indication of cell activity (Fig 3B).

After 4h of exposure to strain S1116, cellular vacuolization was observed in the hindgut, but the epithelium still showed integrity and apocrine secretion activity (Fig 3D); in other specimens, the apocrine secretion increased more and more, and the epithelium integrity remained very conserved in the full extension of the gut (Fig 3A). After 12h, the gut showed highly columnar cells and many more basophilic vesicles

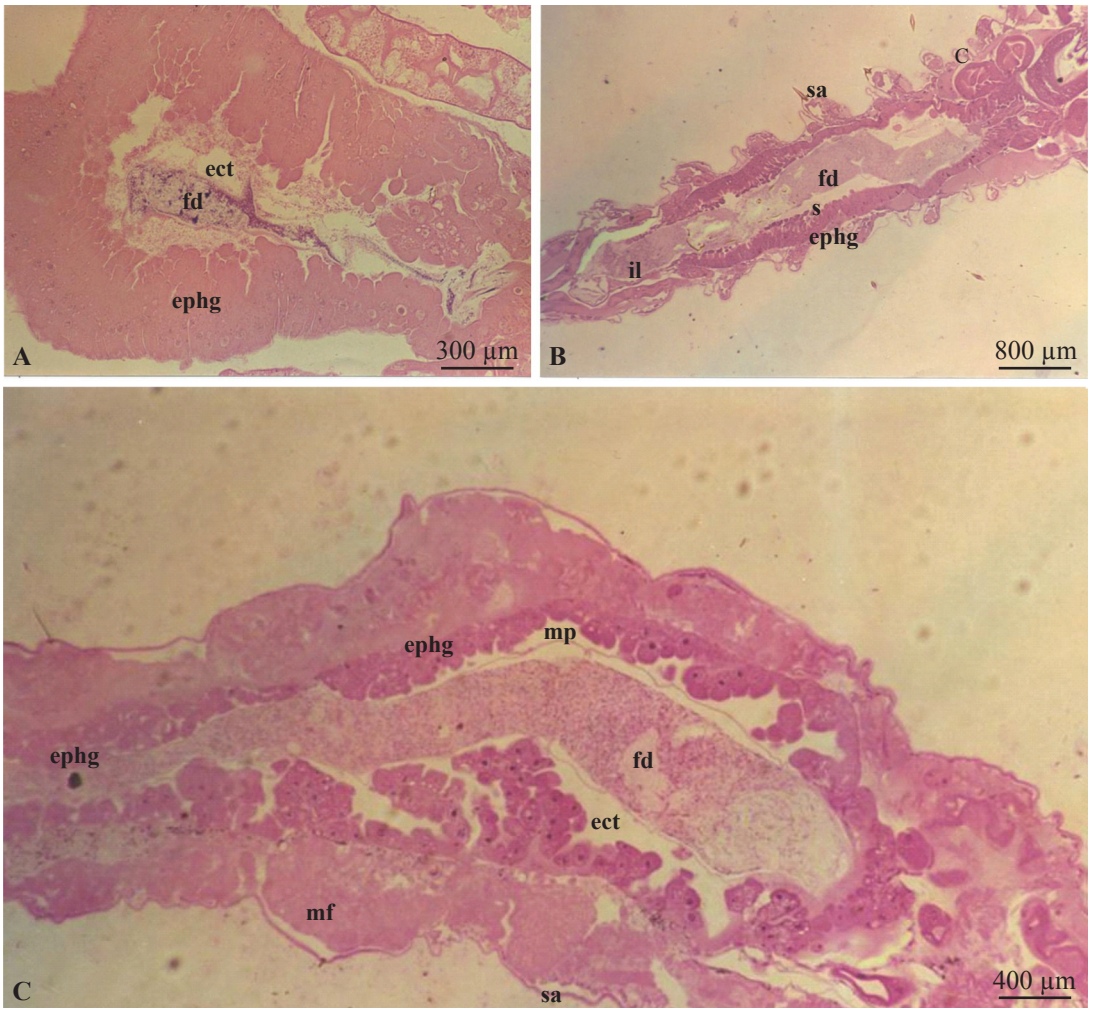

Fig 2 Histological sections of 3rd instars of *Anopheles albitarsis* after exposure to bacilli S1116. (A) Cells of the hindgut after 2h. (B) Larva detail after 4h. (C) Cells of the midgut after 6h. C, cardia; ect, ectoperitrophic space; ephg, epithelium of hindgut; fd, food in gut lumen; il, ileum; mf, muscle fibers; mp, peritrophic membrane; s, secretion; sa, abdominal segment.

migrated to the ectoperitrophic space, where some of them could be observed detaching from the gut epithelial cells (Figs 3A,F). Under exposure to the medium containing strain 2362, the 4th instars showed the same alterations as with strain S1116 (Fig 3F).

The larval epithelial cells of *A. aegypti* 4th instars did not show any injury after 72h exposed to strain 2362 as compared to the control (Figs 4A-D). However, the gut epithelium of larvae exposed to strain S1116 became totally disorganized as early as 15 min to 30 min, or only 4h after exposition. Gut cells were very cubic and separated from each other and from the basal membrane (Figs 3G, 4E,F). The histopathological symptoms observed were cell hypertrophy mainly in the hindgut, increase of apocrine cell secretion, cell vacuolization, cell desegregation, cell lysis, and disorganization of the gut epithelium.

Of all the species studied, *C. quinquefasciatus* 4th instars were the most susceptible to the toxins of both the bacterial strains tested (S1116 and 2362). In both cases, the intestinal epithelium was completely damaged after 1h of exposure to the bacteria (Fig 5 A-D), showing the same histological pattern described for the other species.

In general, the strain S1116, in 15 min the intestinal epithelium was already totally degenerated. Between 15 min and 1h following the exposure to the contaminated medium, the hindgut was already completely damaged, as well as the portion of the foregut and gastric caeca. For bacilli 2362, following 5 min of exposure to the contaminated medium, portions of the fore- and midgut already contained separated cells, with their intercellular links destroyed. A similar situation could also be seen in the larvae following 30 min of exposure.

Discussion

The histopathological process of *Bacillus* infection of mosquito guts is quite similar in all species studied and other dipterans (Davidson 1979, Habib 1983, Karch & Coz 1983, Davidson & Titus 1987, Koua *et al* 1998, Rey *et al*

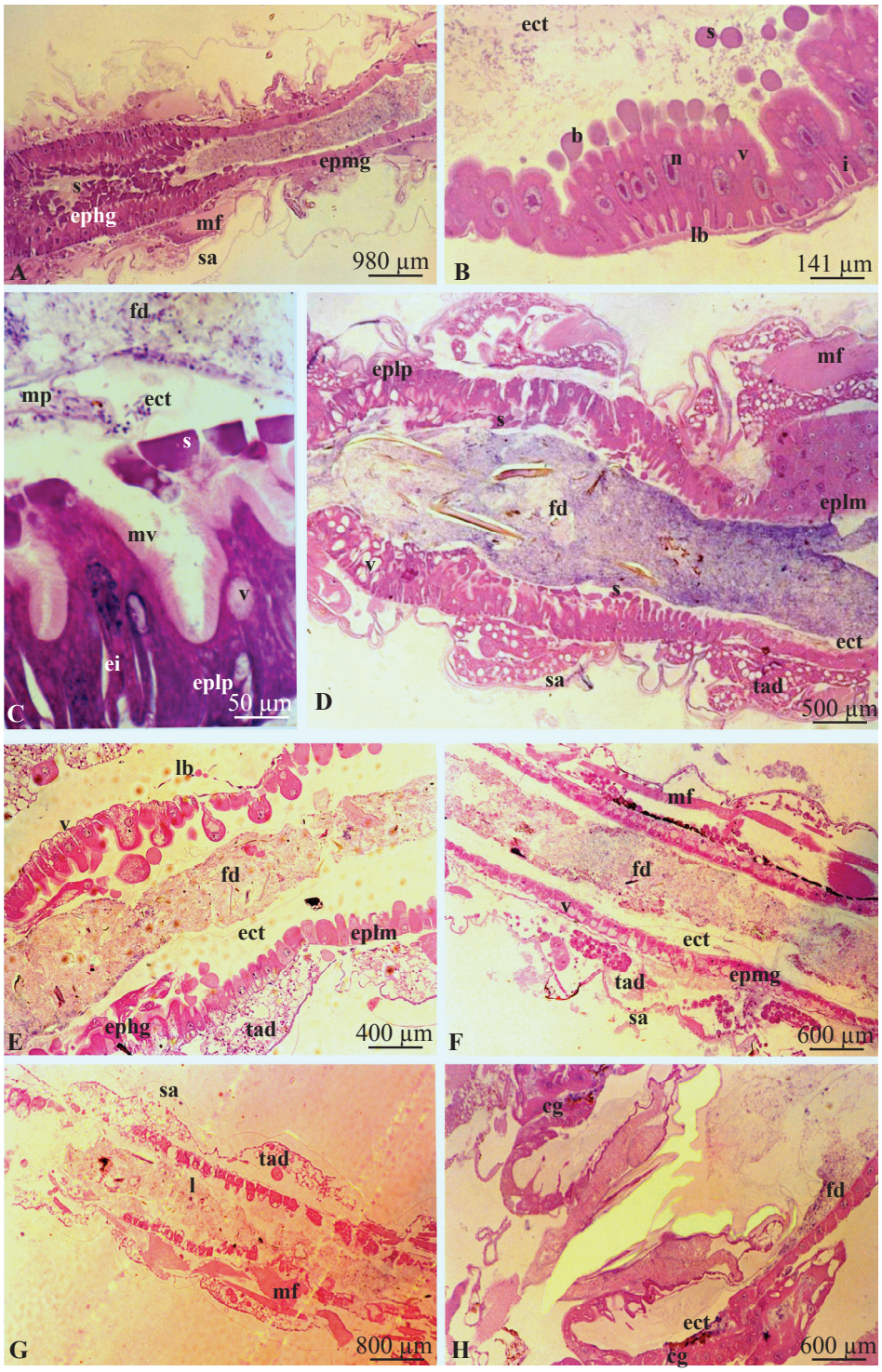

Fig 3 Histological sections of 4th instars of *Anopheles albitarsis* and *Aedes aegypti*, after exposure to *Bacilus sphaericus* strains 2362 and S1116. (A) General view of *A. albitarsis* larva after 12h exposed to bacilli S1116. (B) Cells of the hindgut highly hypertrophied with secretion production of *A. albitarsis* larvae after 30 min exposed to bacilli S1116. (C) Detail of the cells of the hindgut of *A. albitarsis* larvae after 10 min exposed to bacilli 2362. (D) Mid- and hindgut of *A. albitarsis* larvae after 4h exposed to bacilli S1116 (E) Mid- and hindgut of larvae of *A aegypti* after 4h exposed to bacilli S1116. (F) Midgut of *A. albitarsis* larvae after 12h exposed to bacilli 2362. (G) Midgut of *A. aegypti* larvae after 30 min exposed to bacilli S1116. (H) Gastric caecae of *A. albitarsis* larvae after 30 min exposed to bacilli S1116. *; degenerated cells or tissue; b, bubbles; lb, basal lamina; C, cardia; ect, ectoperitrophic space; epfg, epithelium of foregut; ephg, epithelium of hindgut; epmg, epithelium of midgut; fd, food in gut lumen; gc, gastric caecae; i; basal lamina invagination; ie, intercellular space; l, intestinal lumen; mf, muscular fibers; mp, peritrophic membrane; mv, microvilli; n, nucleus; s, secretion; sa, abdominal segment; tad, degenerating tegument.

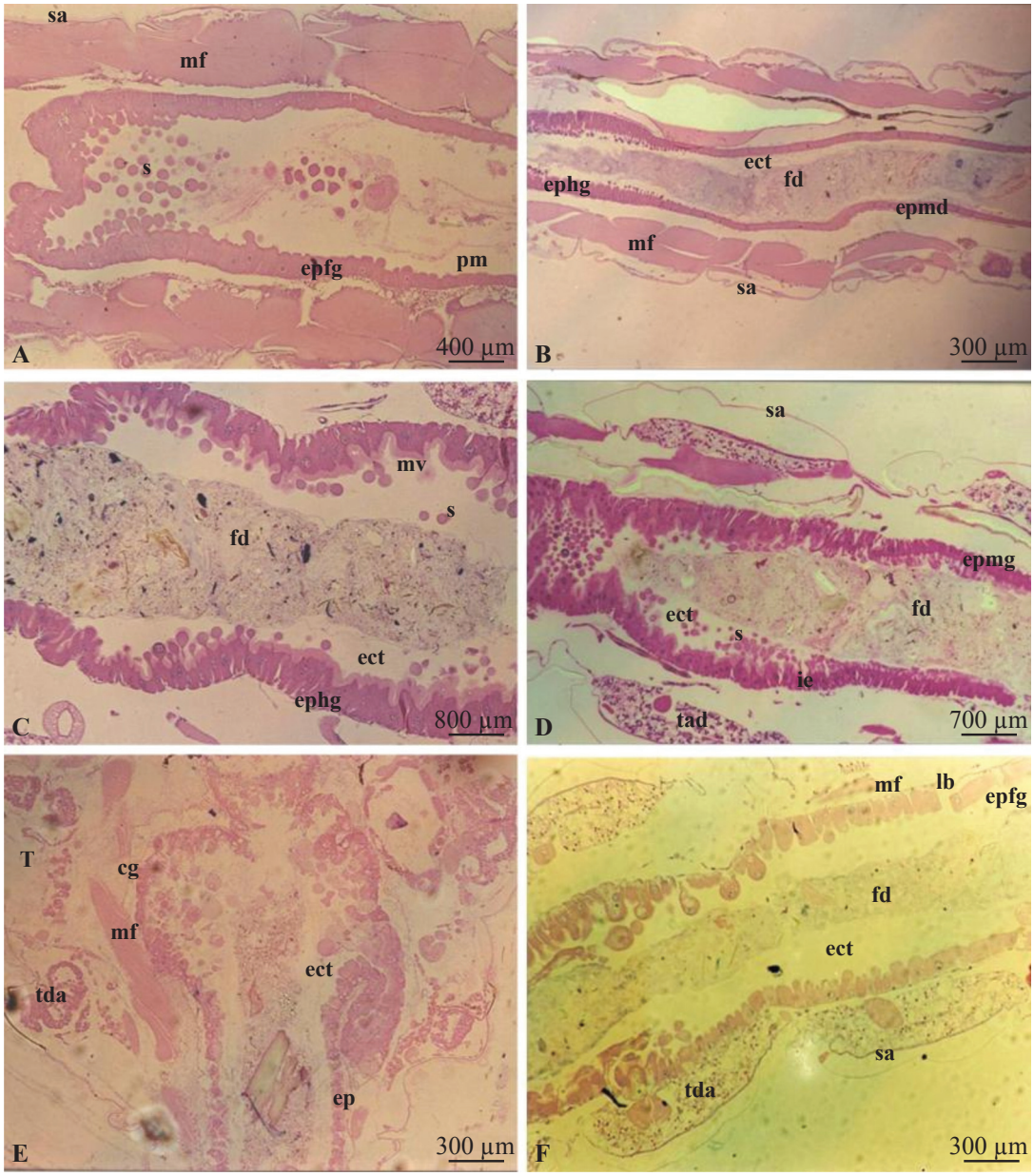

Fig 4 Histological sections of 4th instars of *Aedes aegypti* after exposure to *Bacillus sphaericus* strains 2362 and S1116. (A) Hindgut of the control (B) mid- and hindgut after 15 min of exposure to bacilli 2362. (C) Hindgut after 1h of exposure to bacilli 2362. (D) Hindgut after 72h of exposure to bacilli 2362 (E) Thorax after 15 min of exposure to bacilli S1116. (F) Fore- and midgut after 4h of exposure to bacilli S1116. *, degenerated cells or tissue; b, bubbles; lb, basal lamina; C, cardia; ect, ectoperitrophic space; epfg, epithelium of foregut; ephg, epithelium of hindgut; epmg, epithelium of midgut; fd, food in gut lumen; gc, gastric caecae; i, basal lamina invagination; ie, intercellular space; l, gut lumen; mf, muscle fibers; mp, peritrophic membrane; mv, microvilli; n, nucleus; s, secretion; sa, abdominal segment; tad, degenerating tegument.

1998). The mosquitoes studied showed a common pattern of morphological changes of the gut epithelium throughout the entire digestive apparatus. In all analyzed species, the first histopathological symptom of the activity of bacterial toxins was the hypertrophy of the hindgut, which although observed in control groups, was much less conspicuous. Depending on the culicid species and on bacterial strain used (2362 or S1116), the hypertrophy of the foregut took place from 5 min to 15 min after exposure to the contaminated medium. The increased apocrine secretion observed in infected mosquitoes is also found in vertebrate mammary glands. An intestinal cell-secretion-releasing mechanism such as in the dipterans is not found in vertebrates. Digestive-system glands usually secrete through a process called merocrine (exocytose). In vertebrates, the absorptive epithelial cells (which bear the microvilli) and the mucus cells (or goblet cells, which have no "brush border" or microvilli in their apical portion) do not show this type of secretion-releasing mechanism either.

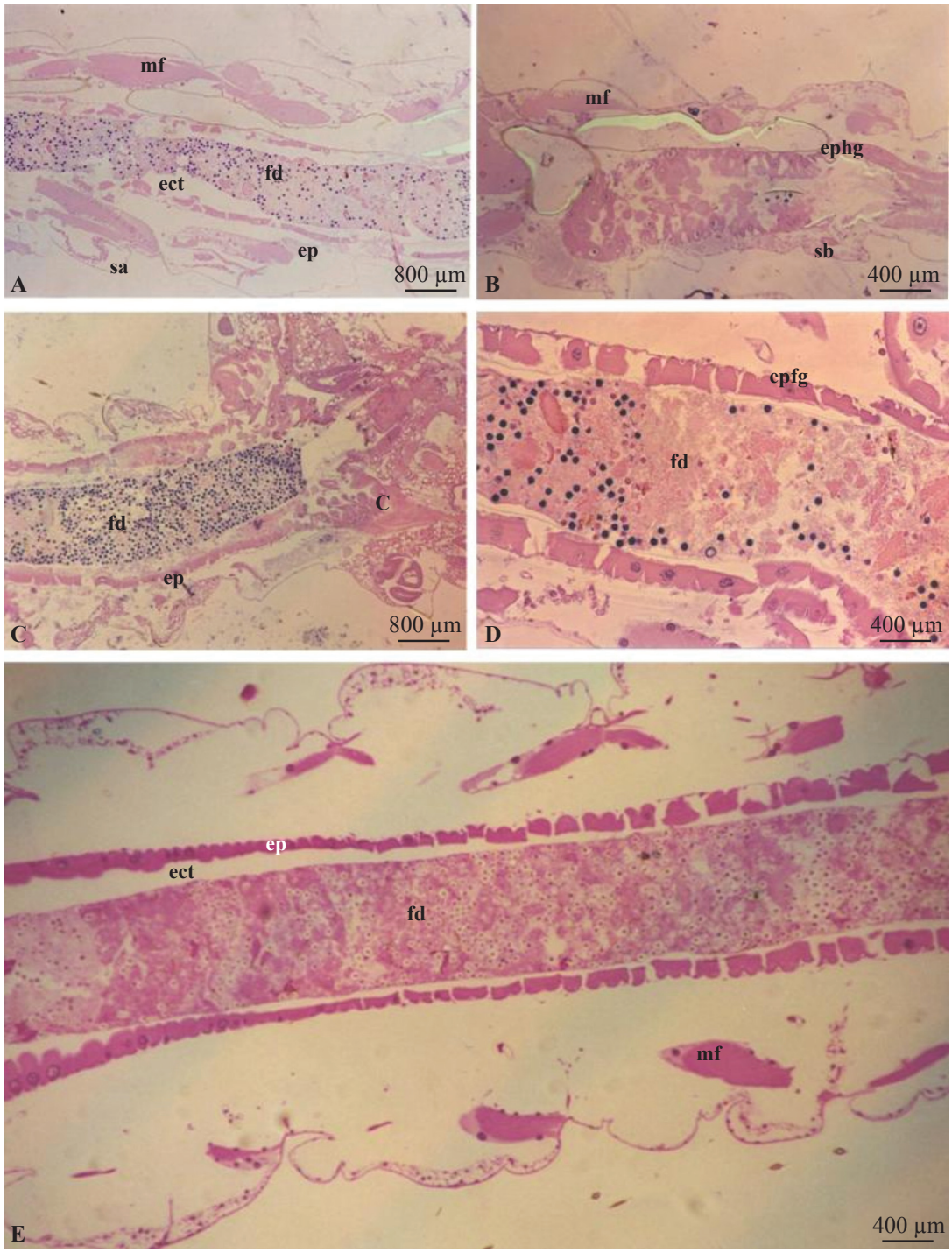

Fig 5 Histological sections of 4th instars of *Culex quinquefasciatus* after exposure to *Bacillus sphaericus* strains 2362 and S1116. (A) Midgut after 15 min of exposure to S1116. (B) Hindgut after 15 min of exposure to S1116. (C) Foregut after 1h of exposure to S1116 (D) Foregut after 5 min of exposure to 2362. (E) Midgut after 30 min of exposure to 2362. C, cardia; ect, ectoperitrophic space; ep, gut epithelium; epfg, epithelium of foregut; ephg, epithelium of hindgut; fd, food in gut lumen; mf, muscle fibers; sa, abdominal segment.

There are no studies on how and why this process takes place in invertebrates. Some authors have reported that cells lose their microvilli due to bacterial endotoxins, as they can affect the cytoskeleton. Cytoskeleton disorganization may aid in cell blebbing. However, according to our findings, it is quite clear that cells that secrete vesicles lose their microvilli. The chemical nature of the vesicle is unknown. We cannot explain why vesicles result as a reaction against the

bacterial endotoxin, or unsure if these vesicles are a response to the endotoxin or a product of the endotoxin damage to the cytoskeleton organization. However, there are indirect indications that this mechanism of secretion may be related to a defense mechanism, as apocrine secretion is more intense in the most resistant species.

In invertebrates, mainly in the Hymenoptera, Caetano *et al* (1994) reported the apocrine secretion in the epithelium of the ventricle of adults of the ant *Pachycondyla striata* (Fr. Smith). The secretion-releasing process is basically the same as the one reported in here. However, in *P. striata,* the portion of the cytoplasm released to the peritrophic space is often accompanied by the microvilli, forming vesicles with brush-border traces. These vesicles are able to pass through the peritrophic membrane to the gut lumen, where they undergo lysis, with the consequent release of their contents (Caetano et al 1994).

The analysis of the content of the apocrine secretion in adult honeybees indicated they are positive for acid phosphatase, which may indicate that the vesicles may have lysosomal content and be used in the pollen digestive process in honeybees (Cruz-Landim et al 1996).

This morphological study of the gut changes of vector disease mosquitoes under *Bacillus* toxins is the first to suggest the apocrine secretory activity in Culicidae as defense answer agains the *Bacillus* infection, but much more studies about this aspect must be carried out.

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