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Comparative behavioural and anatomo-pathological investigations on *Musca domestica* L. adults treated with a new strain of

ABSTRACT

Comparative behavioural bioassays and anatomo-pathological investigations have been carried out on *Musca domestica* adults to test insecticidal activity of a new isolate of *Bacillus* sp. (characterization in course) close related to *B. thuringiensis* (Berliner). Both behavioural bioassays and anatomo-pathological tests, parallel the results previously reported for many other insects similarly treated with different strains of the well known *B. thuringiensis.* Flies fed on a suspension of a *Bacillus* sp. new isolate sporulated culture, display progressive sluggish and shaky behaviour, decreased responsiveness to external stimuli, gradual feeding inhibition, and general paralysis, until they die (over 52% within 72 hrs, and almost all specimens within 4-5 days, after treatment). Anatomo-histological and ultrastructural investigations corroborate the results from behavioural bioassays, proving that flies treated with *Bacillus* sp. undergo progressive degeneration (up to rupture) of midgut and malpighian tubules' epithelial cells, as well as of the midgut muscular sheath, as reported for other insect species treated with *B. thuringiensis*. Comparative tests on *M. domestica* adults treated with analogous suspension of *B. th*. var. *kurstaki* (HD-1) gave rather negative results.

Key words: behavioural bioassays, electron microscopy, gut anatomy, insecticidal activity, midgut histology, light microscopy.

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1. INTRODUCTION

The house fly (*Musca domestica* L.) is a well-known saprophagous insect, usually considered as an annoyer and potential carrier of disease-producing microorganisms. Hence, due to its usual association with human and livestock habitats, it is of considerable medical and veterinary importance. For these reasons, together with its great reproductive potential, the house fly control is almost necessary.

According to the successful microbiological control experience using *Bacillus thuringiensis* (Berliner) on lots of injurious insects, the availability of an appropriate strain of *B. th.*, or another related bacterial species, might be a convenient control measure of house fly.

Bacillus thuringiensis (as well as other *Bacillus* species like, e.g., *B.* sp*haericus* or *B. popilliae*) is a sporeforming bacterium which produces different insecticidal toxins, such as endotoxins, exotoxins, haemolysins or enterotoxins (GLARE and O'CALLAGHAN, 2000). Toxicity of individual *B. th.* strains is dependent on the number and type of toxins they do produce.

During sporulation, *B. th.* produces parasporal crystalline inclusions containing proteins called δ-endotoxins which, after being ingested by the insect, are activated in the midgut and interact with the epithelium, causing disruption of the epithelial cells and eventually insect death (GILL *et al.*, 1992).

Similarly, other related bacterial species as *B.* sp*haericus* produce spores and crystals containing proteins toxic to dipteran larvae.

Bacillus thuringiensis crystal proteins, better known as Cry toxins, normally display a very specific activity on particular insect species. On the contrary, cytolitic endotoxins (or Cyt toxins) have a wider spectrum of activity and are deposited together with Cry toxins in the crystalline inclusion bodies in *B. th. var. israelensis* strains used for Diptera control.

During its development, *B. th.* may release in the nutrient medium other

metabolites having insecticidal properties. The best known metabolite is a thermostable and water soluble β-exotoxin, also called fly-toxin or thuringiensin, which possesses a broad-spectrum of insecticidal activity. Different isolates of *B. th*. may or may not produce this exotoxin, depending on the strain as well as on the fermentation media used for their production (MOHD-SALLEH and BEEGLE, 1980).

Several researchers have found synergism between β-exotoxins and δ-endotoxins (DUBOIS, 1986; MOAR *et al*., 1986).

More recently a new class of insecticidal proteins with a wider toxic activity, produced during the vegetative phase of some *B. th.* strains (vegetative insecticidal proteins or VIP), have been identified, as well as Mtx toxins have been identified in *B. sphaericus*.

Therefore, entomopathogenic bacteria produce a large variety of toxins with different mechanism of action (MAAGD *et al*., 2003).

It is generally accepted that *B. th*. activated toxins, after binding specific plasma membrane receptors on the midgut epithelial cells, generate leaky pores in the plasma membrane of susceptible cells which, due to the consequent inflow of ions and water, eventually undergo a colloid-osmotic lysis.

The biotoxic action of *B. th*. δ-endotoxins produced as crystal proteins during sporulation has been widely investigated (KNOWLES and ELLAR, 1987), and a lot of work has been carried out on their histopathological effects (such as progressive deterioration of epithelial cell cytoplasm, especially consisting of: microvillar disruption, endoplasmic reticulum remarkable vacuolation, mitochondrial degeneration, excessive number of lysosome-like structures, loss of ribosomes, rough endoplasmic reticulum disorganization, disappearance of basal infoldings, up to cell rupture; as well as deterioration of midgut muscular sheath and related connective tissue) on many insect species belonging to the principal taxonomic groups such as Lepidoptera (ENDO and NISHIITSUTSUJI-UWO, 1980; PERCY and FAST, 1983; SPIES and SPENCE, 1985; MATHAVAN *et al*., 1989; LANE *et al*., 1989; RAUSELL *et al*., 2000), Coleoptera (BAUER and PANKRATZ, 1992) and Diptera (including *M. domestica*) larvae (LACEY and FEDERICI, 1979; LAHKIM-TSROR *et al*., 1983; SINGH *et al*., 1986; Weiser and ZIZKA, 1994), as well as Rhyncota (MADDRELL *et al*., 1988) and adults of Mallophaga (HILL and PINNOCK, 1998) and Orthoptera (QUESADA-MORAGA and SANTIAGO-ALVAREZ, 2001).

About the pathological effects of *B. th*. on Diptera, investigations have been carried out on Simulidae (LACEY and FEDERICI, 1979) and Culicidae (WEISER and ZIZKA, 1994) larvae. Concerning *Musca domestica*, dramatic effects of *B. th*. var. *israelensis* δ-endotoxins on the larvae neuromuscular system were proved by an *in vitro* experiment (SINGH *et al*., 1986).

Generalized myotoxic and neurotoxic effects (with decreased responsiveness to external stimuli, obvious sluggish behaviour and general body paralysis) by the same endotoxins have been proven also on Blattodea (SINGH and GILL, 1985).

However, little work has been done with adult Diptera (YOUNES *et al.*, 1996) and, as far as we know, no investigations have been carried out yet on the insecticidal activity of *B. th.* or other *Bacillus* species on the house fly adults.

Our aim was to test for biotoxic action on an hygienically and economically important insect such as *M. domestica* adult, a sporulated culture of a new isolate of *Bacillus* sp., close related to *B. thuringiensis*, as well as its culture supernatant, both contrasted with *B. th.* var. *kurstaki* (HD-1) spore-crystal suspension, through behavioural bioassays, together with anatomical, histological and ultrastructural investigations on the midgut and malpighian tubules of treated flies, compared with healthy ones.

2. MATERIALS AND METHODS

2.1 INSECTS

A *M. domestica* strain from the Entomology Institute of Milan University, then continuously cultured for about two years at the Entomology Section of the Department of Plant Protection of Sassari University, was used for all investigations (FLORIS *et al.*, 2002).

Groups of newly-emerged adults, four replicates of 10 flies each, were kept in plastic boxes with a transparent cover, in a cabin (24-25°C, 64-65% R.H., 12-h photophase/scotophase regimen) at the Entomology Laboratory of the Department of Arboriculture and Plant Protection of Perugia University, to be especially used for microscopical investigations; as well as at the Section of Entomology of the Dep. of Plant Protection of Sassari University, to be mainly used for behavioural bioassays. Samples to test were fed daily on: (1) a spore suspension of a new isolate (see below) of *Bacillus* sp. ($2x10^9$ spores/ml), or (2) its culture supernatant, or (3) analogous suspension of *B. thuringiensis* var. *kurstaki* (HD-1), each diluted 1:1 with a 60% sucrose solution in distilled water, presented to flies in capillary tubes. Control samples were fed on 30% sucrose water solution.

2.2 BEHAVIOURAL OBSERVATIONS

Behavioural observations were daily conducted (at 24 hrs, 48 hrs, 72 hrs after fly emergence) on both, treated and control flies, through direct watching.

Fly mortality was assessed every day and data were normalized by an arcsine-square-root transformation and analysed by a one-way ANOVA followed by Dunnett t-tests to

compare differences among the various treatments and the control (SPSS 11.0 for Mac OS X Standard Version).

2.3 Light microscopy

In order to study intoxication effects over time, several specimens from each of the four fly samples were sacrificed at 24 hs, 48 hs or 72 hs after emergence and (or not: controls) being treated daily (see above: Insects).

Insects were always anaesthetised by appropriate low temperatures, before being dissected.

For light microscopy investigations, specimens were dissected in saline and directly observed in fresh whole mounts at a stereo or compound microscope, or segments of midgut and malpighian tubules were processed and embedded in Epon-Araldite (as below reported for transmission electron microscopy), and sections 1 µm thick, cut with an L.K.B. "Nova" ultramicrotome and normally stained with Methylene Blue, were examined and micrographed through a Zeiss III Photomicroscope.

2.4 Electron microscopy

For scanning electron microscopy (S.E.M.) observations, the whole intestine or just the midgut of treated or non-treated (control) adults were fixed for 3 hs in Karnovsky's (1965) fixative solution, post-fixed for 1 h in 1% Osmium tetroxide, dehydrated in a graded ethanol series and then immersed in Hexamethylene-disilazane until the latter was completely evaporated. Then they were mounted on normal specimen holders, gold/palladium coated in a Balzers Union SCD 040 sputter unit and examined and micrographed with a Philips XL30.

For transmission electron microscopy (T.E.M.) investigations, midgut portions close to the pyloric valve, as well as of malpighian tubule upper segments, were excised from living anaesthetized flies sacrificed 24 hs, 48 hs or 72 hs after emergence and (or not: controls) being daily treated, and immediately immersed in Karnovsky's (1965) fixative solution. After a quite long (72 hs) fixation at 4°C, the specimens were rinsed overnight in cacodylate buffer with 5% sucrose, then post-fixed in 1% Osmium tetroxide for 1 h, rinsed again in cacodylate buffer, dehydrated in a graded ethanol series until 90%, then block stained with 1% uranyl acetate in 95% ethanol solution for 1 h, next two 15 min passages in absolute alcohol and finally embedded through propylene oxide in Epon-Araldite. Sections about 70 nm thick, cut with an L.K.B. "Nova" ultramicrotome, sequentially stained with uranyl acetate and lead citrate, were finally examined and micrographed through a Philips EM 400T.

2.5 Bacteria

For our investigations, a spore suspension of a new isolate of *Bacillus* sp., close

related to *B. thuringiensis* (identification still in progress), or its culture supernatant, or an analogous suspension of *B. th*. var. *kurstaki* (HD-1) were used.

The new isolate of *Bacillus* sp., collected in Sardinia from a soil near Oliena and Orotelli (towns in the Nuoro Province), was grown in culture according to a somewhat modified method developed by KARAMANLIDOU *et al*. (1991). Bacteria were cultured for 5 days in flasks containing 200 ml of nutrient broth at 30°C and 220 rpm until sporulation of the majority of the bacterium population. The whole sporulated culture was harvested by centrifugation (at 10000 rpm for 10 minutes at 4°C), washed three times in sterile water, and resuspended in water to obtain a suspension (below mentioned as spore suspension) with a final concentration of about $2x10^9$ spores/ml.

Culture supernatant was saved for bioassays since it could contain some soluble toxic compounds. No significant mortality was observed in preliminary bioassays when this fraction had been autoclaved (121°C per 15 minutes) to test for the presence of heat-stable toxins. While, the supernatant fraction used in bioassays for the histopathological observations was not heat treated to preserve eventual soluble compounds released in the medium. Moreover, phase microscope observation of this fraction, evidenced the presence of a few spores.

B. th. var. *kurstaki* strain HD-1 was cultured in the same way as the *Bacillus* sp., and with the same method (see above) was prepared its spore-crystal suspension.

3. RESULTS

3.1 FLIES BEHAVIOUR

Behavioural observations on flies treated with *Bacillus* sp. new isolate allow to distinguish the following four successive phases developing from the first administration (at fly emergence) of the spore suspension up to death of treated flies:

- 1st phase (24 hrs after emergence and being treated): flies do not show any intoxication specific symptoms, but some of them do not look as active as untreated (control) ones; no mortality is found with treated or control samples within 24 hrs, while the first symptoms of intoxication (see below) appear only in few treated flies 36 hrs after being treated;
- 2nd phase (48 hrs after emergence and being daily treated): only some flies show sluggish and shaky behaviour and decreased responsiveness to external stimuli but they continue to feed although at a rate lower than control ones; mortality appears in all of the samples but at significantly (tab. 1) higher degree in the specimens fed with spore suspension of *Bacillus* sp. only;
- 3rd phase (72 hrs after emergence and being daily treated): over 52% of the flies die, while most of remaining specimens stop feeding and undergo general paralysis (often

lying on the back); whereas control flies still look normal;
- 4th phase (within 1-2 days later on): almost all treated flies gradually die.

Flies treated with *Bacillus* sp. supernatant show a non significant mortality level (tab. 1) with no behavioural symptoms of intoxication compared to the samples treated with *Bacillus* sp. spore suspension.

Flies treated with *B. th.* var. *kurstaki* suspension still behave normally 72 hrs after emergence and being daily treated, just like control.

Table 1

	Flies mortality % ± s.d.	
	After 48 hrs	After 72 hrs
Bacillus sp. (spore suspension)	25.0 ± 12.9*	52.5 ± 17.1*
Bacillus sp. (culture supernatant)	5.0 ± 5.8	15.0 ± 5.8
B. th. var. *Kurstaki* (spore-crystal suspension)	0.0 ± 0.0	2.5 ± 5.0
Control	2.5 ± 5.0	5.0 ± 5.8

(asterisk * indicates significant ($P<0.05$) difference in mortality from control, on the 95% confidence interval)

3.2 Pathological effects on alimentary canal and malpighian tubules at gross anatomy level

The adult house fly alimentary canal (fig. 1) consists of: a foregut (stomodaeum) having a narrow, long oesophagus with a large discrete ingluvies at posterior end, just before entering a conspicuous, mushroom-shaped cardiac valve (fig. 1: CV); a very long and convoluted midgut (mesenteron: fig. 1: MG) having a quite strong muscular sheath (figs 2, 3a, 8); and hindgut (proctodaeum) displaying well distinct ileum and colon (fig. 1: IL, CO) and rectum (fig. 1: RC).

In the fresh whole mounts, under a stereo microscope, it is possible to detect only one clear difference between healthy flies (control) and specimens treated with *Bacillus* sp., i.e., a sort of peristalsis disruption or gut paralysis, which appears in the midgut after 48 hrs (or later) by a discontinuity in the alimentary bolus which looks divided into pellets of different size, while the corresponding outer surface of the midgut shows moderate irregular swellings.

The malpighian tubules (figs 2 and 11) are formed each by two distinct segments: a lower segment (ureter) debouching into the gut lumen close to the pyloric valve, and an upper one having two branches connected to each other and with the lower segment distal end as to form a T shape (fig. 11).

No differences with malpighian tubules between healthy and treated flies can be detected in the fresh whole mounts at a stereomicroscope or at a scanning electron microscope.

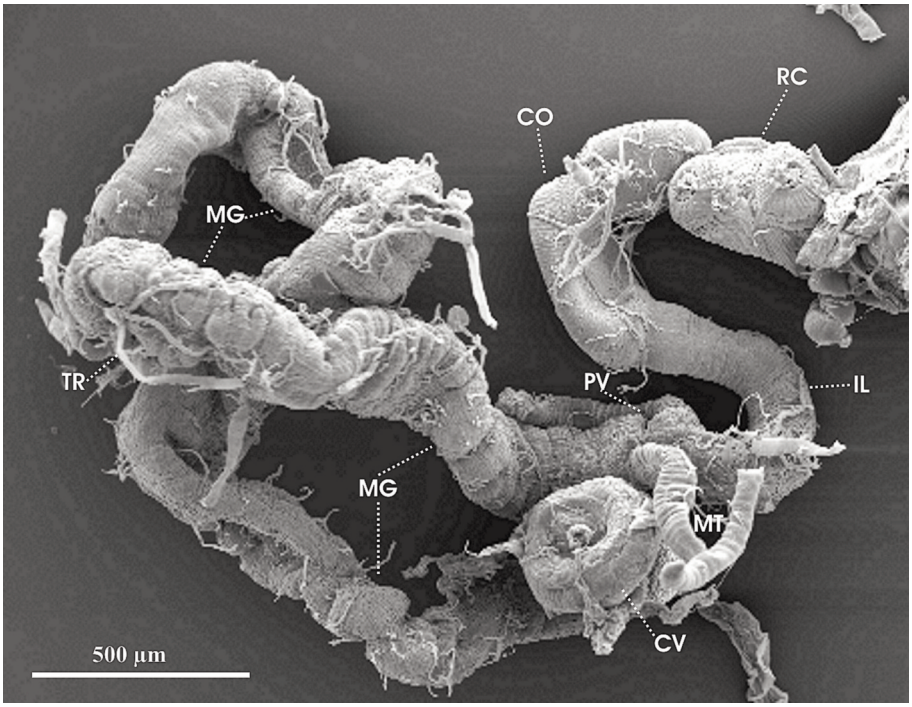

Fig. 1 - *Musca domestica*, adult female 24 hrs old. Scanning electron (S.E.M.) micrograph of alimentary canal, general view of midgut (mesenteron) and hindgut (proctodaeum). CV, cardiac valve; CO, colon; IL, ileum; MG, midgut; MT, malpighian tubules; PV, pyloric valve; RC, rectum; TR, tracheae.

3.3 HISTOPATHOLOGICAL EFFECTS

3.3.1 Light microscopy observations

Preliminary observations with light microscopy on cross sections already show some of the attended intoxication effects (then confirmed by scanning and transmission electron microscopy) on the midgut epithelium of specimens sacrificed 24 hrs (or more) after treatment, i.e., a remarkable extended vacuolation of cytoplasm (fig. 3b) and an obvious deterioration of the midgut muscular sheath (fig. 3, b and c).

3.3.2 Electron microscopy observations

Midgut epithelial cells, as well as the midgut muscular sheath and relative connective tissue of healthy specimens (controls) do not show any notable ultrastructural

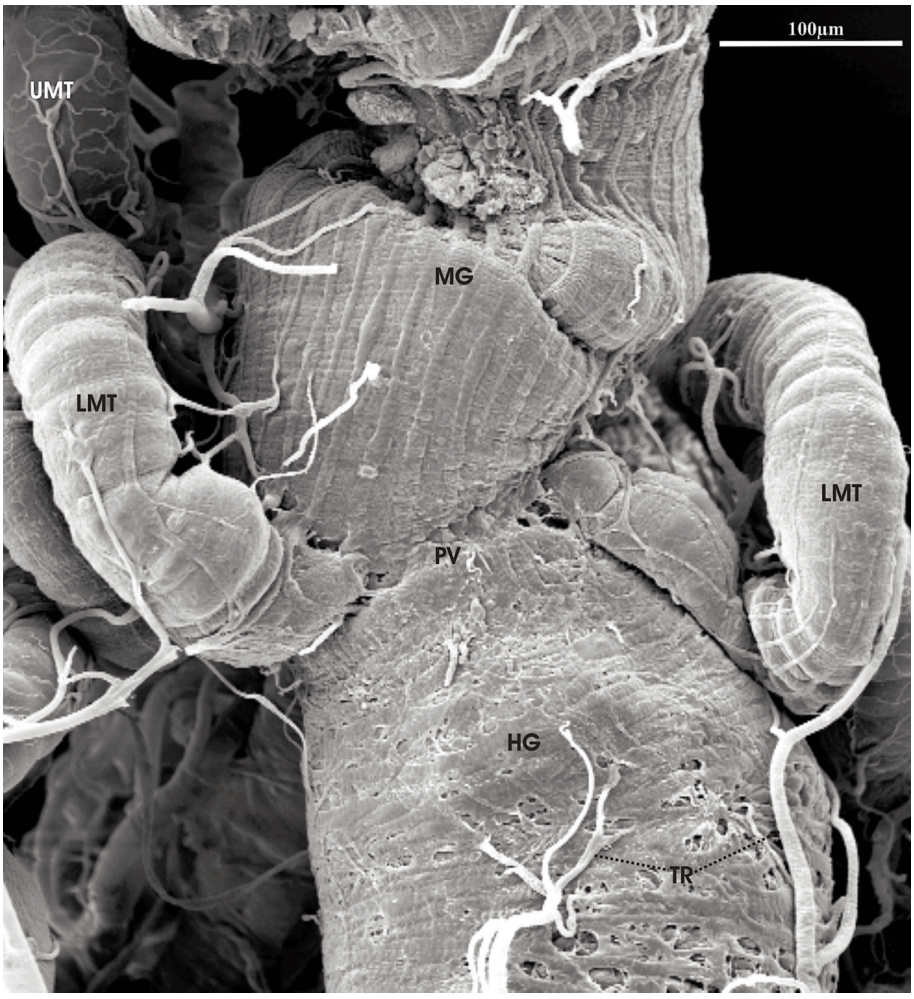

Fig. 2 - Detail of fig. 1, showing pyloric valve region and malpighian tubules. Symbols as in fig. 1, and: HG, hindgut; LMT, malpighian tubule lower segment; UMT, malpighian tubule upper segment.

differences among the samples sacrificed 24 hrs or 48 hrs or 72 hrs after emergence (i.e., after starting of the experiment), both with scanning or transmission electron microscopy observations (figs 2 and 4).

The same ultrastructural condition has been found with malpighian tubules of the above mentioned healthy samples (controls: figs 11 and 12a).

In particular, the midgut epithelial cells of healthy flies (fig. 4) display: apical region

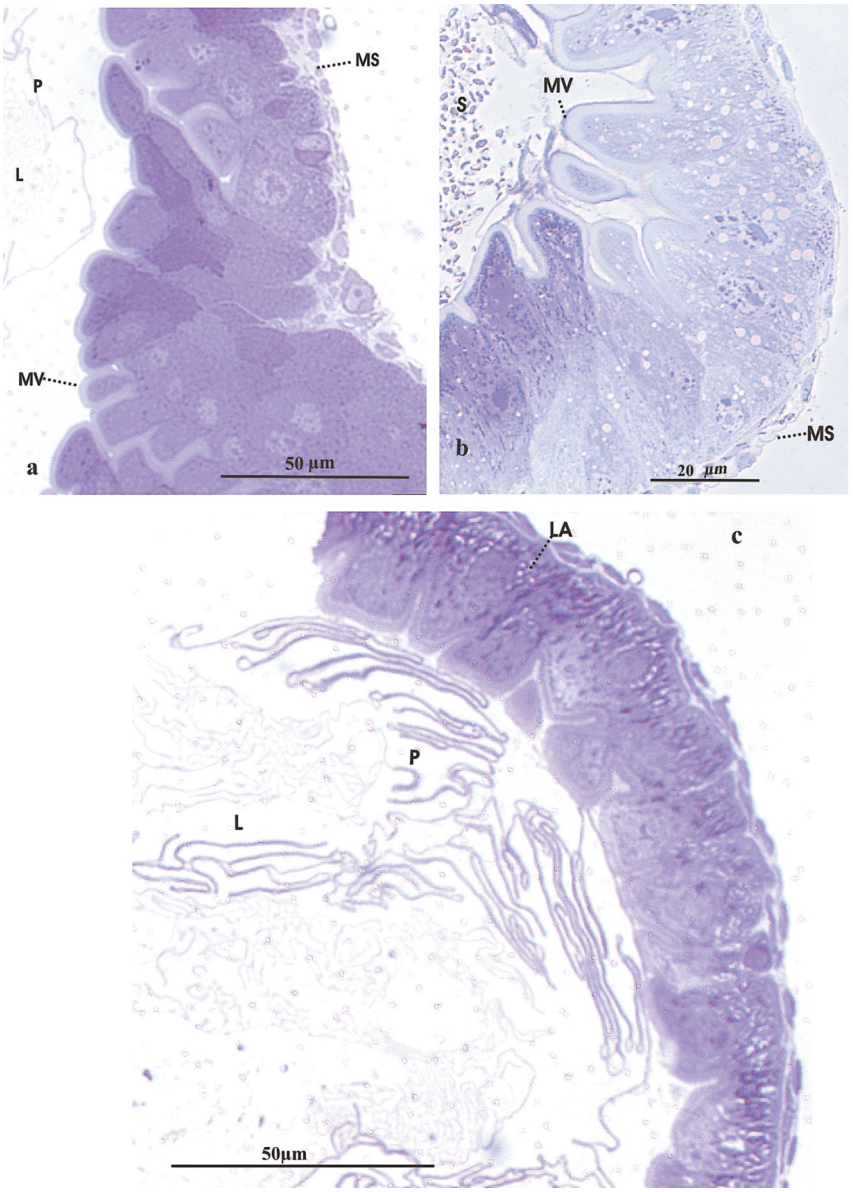

Fig. 3 - *Musca domestica* adult female. Light micrographs of cross sections from: a, healthy specimen 24 hrs old (control); b, specimen sacrificed 24 hrs after emergence and being fed on spore suspension of *Bacillus* sp. new isolate; c, specimen sacrificed 48 hrs after emergence and being fed on *Bacillus* sp. culture supernatant. Note on b and c, midgut epithelial cell obvious vacuolation, as well as muscular sheath deterioration. L, midgut lumen; LA, labyrinth; MS, muscular sheath; MV, microvilli; P, peritrophic matrix; S, spores-parasporal bodies.

provided with well developed and densely packed microvilli projecting into the gut lumen; endoplasmic reticulum quite dense (not showing remarkable vacuolation), mitochondria normally shaped and very numerous especially in the apical region as well as associated with basal infoldings (labyrinth); moderate numbers of lysosome-like structures; abundant ribosomes both free or associated with membranes; well organized rough endoplasmic reticulum; basal labyrinth very well developed and apically extended even beyond the perinuclear region; nucleus quite large and positioned rather posteriorly.

The midgut muscular sheath, made of well developed circular and longitudinal muscular fibrils (figs 2 and 4b), appears quite strong; and the connective tissue binding the muscular sheath to the midgut epithelium basal lamina looks well developed and compact (fig. 4b).

The midgut epithelium continuously produces and releases into the gut lumen a multi-layer peritrophic matrix (fig. 4a: P).

The malpighian tubules (upper segment) of the above mentioned healthy samples, display epithelial cells (fig. 12a) having: apical membrane with well developed and packed microvilli, most of which containing mitochondria; most of cytoplasm occupied by smooth endoplasmic reticulum displaying quite large cisternae; numerous and well shaped mitochondria; nucleus very large and very irregularly shaped as to indicate its highly intense activity; no basal infoldings have been identified. Urate granules are usually present in the tubule lumen (fig. 12a).

In flies sacrificed 24 hrs after emergence and being fed on the spore suspension of *Bacillus* sp., the only notable pathological features consist of a remarkable cytoplasm vacuolation in the midgut epithelial cells, both on cell apical and basal regions (fig. 5, a and b), and an obvious deterioration of the midgut muscular sheath together with its related connective tissue (fig. 5b). Whereas for the rest (epithelial cell microvilli, mitochondria, rough endoplasmic reticulum, nucleus, etc.), the midgut epithelium still looks normal.

In flies sacrificed 48 hrs after emergence and being fed on the spore suspension of *Bacillus* sp., many more and extensive pathological alterations can be detected (fig. 6), i.e., midgut epithelial cells display: most microvilli apparently disrupted; increased population of lysosome-like structures; many mitochondria rather swollen and less electron dense, while others still display cristae and outer membrane apparently normal; rough endoplasmic reticulum rather upset.

It is remarkable that not all the midgut epithelial cells respond to the treatment homogeneously. There are frequently normal cells adjacent to obviously intoxicated ones (fig. 6, lower right corner).

In flies sacrificed 72 hrs after emergence and being fed on the spore suspension of *Bacillus* sp., the whole cytoplasm of intoxicated cells appears dramatically deteriorated (fig. 7a), i.e.: microvilli almost completely disrupted; most of mitochondria deeply

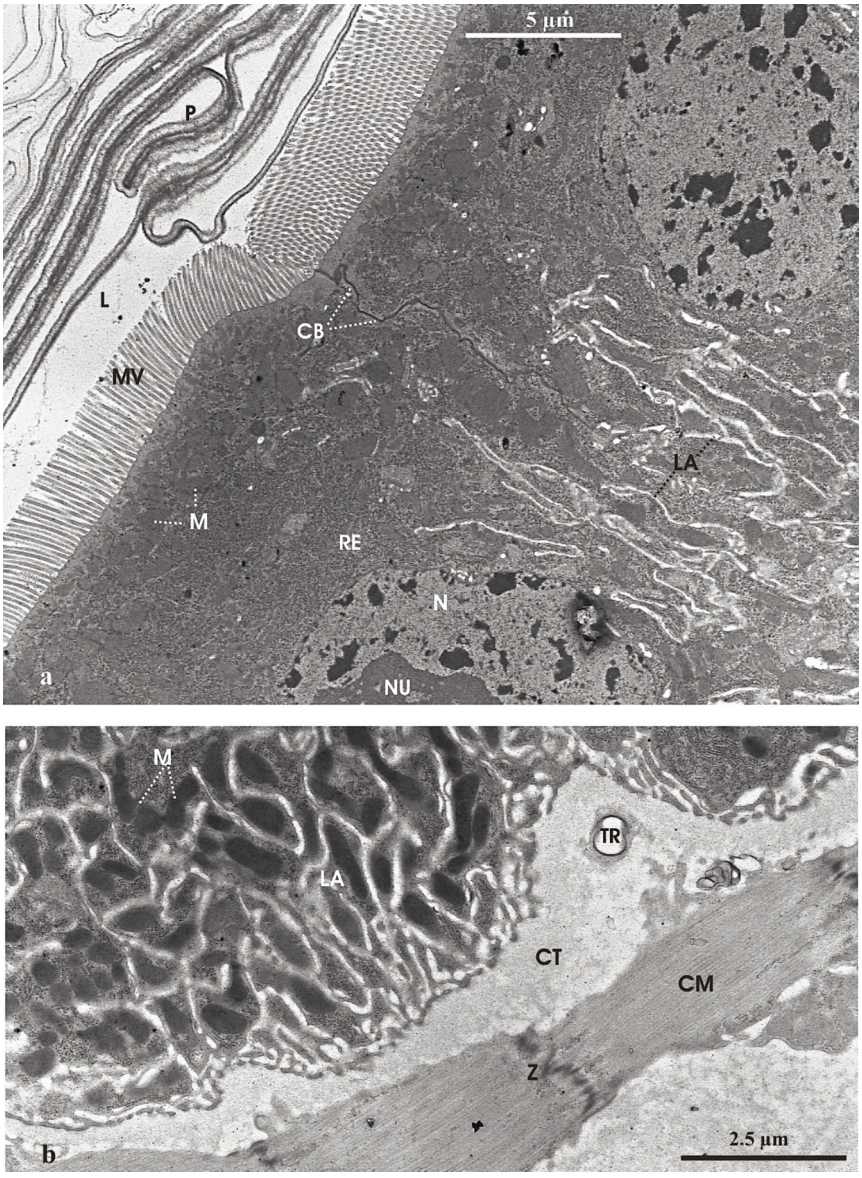

Fig. 4 - Same sample as fig. 3a, i.e., healthy specimen 24 hrs old (control). Transmission electron micrographs from cross section of midgut, showing details of: a, epithelial cell apical and perinuclear aspects, and multilayer peritrophic matrix (P); b, same cell basal aspect, and muscular sheath. CB, cell boundaries; CM, muscular sheath circular fibril; CT, connective tissue; L, midgut lumen; LA, basal labyrinth; M, mitochondria; MV, microvilli; N, nucleus; NU, nucleolus; RE, rough endoplasmic reticulum; TR, tracheole; Z, z-disc.

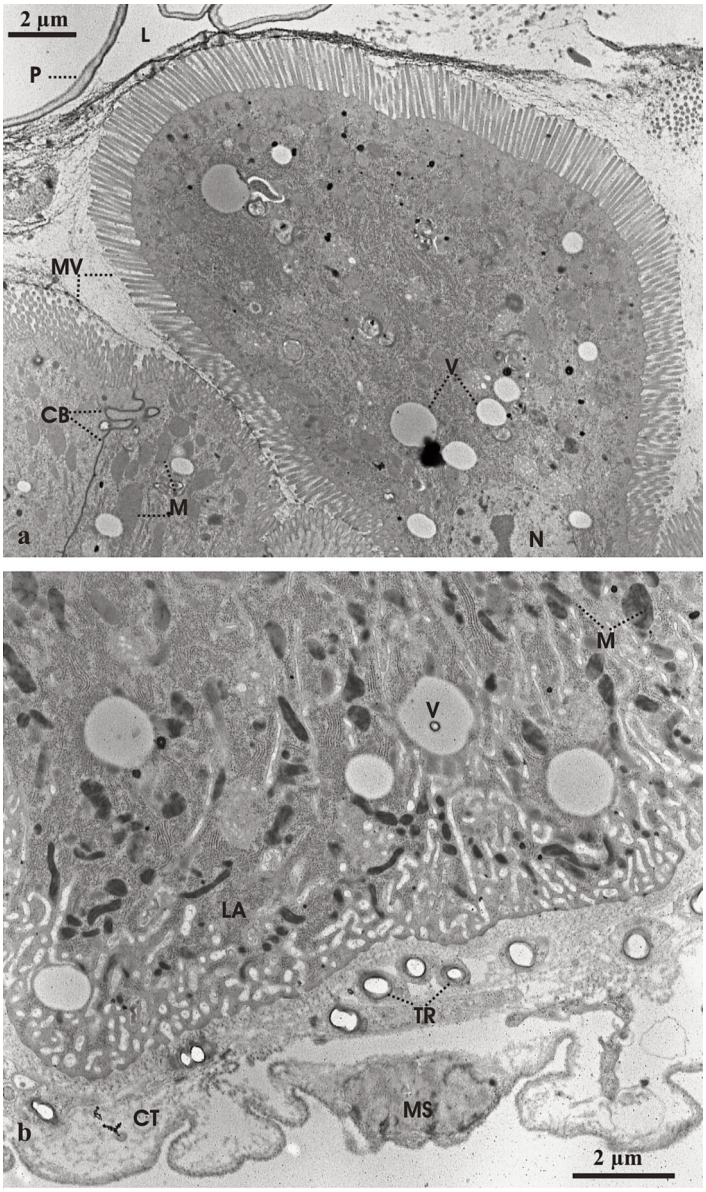

Fig. 5 - Same sample as fig. 3b, i.e., specimen sacrificed 24 hrs after emergence and being fed on spore suspension of *Bacillus* sp. new isolate. Transmission electron micrographs from cross section of midgut, showing cytoplasm vacuolation present on both apical (a) and basal (b) region, as well as muscular sheath and connective tissue deterioration. Symbols as in Fig. 4, and: MS, muscular sheath; V, vacuoles.

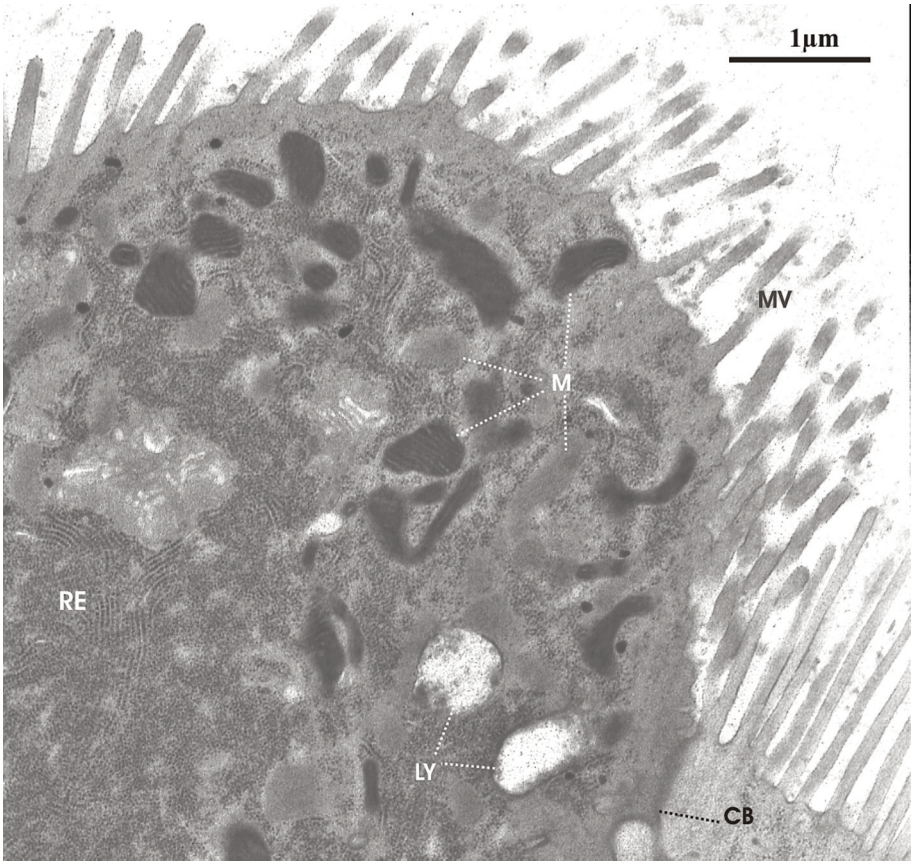

Fig. 6 - *Musca domestica* adult female sacrificed 48 hrs after emergence and being fed on spore suspension of *Bacillus* sp. new isolate. T.E.M. micrograph, section detail of midgut epithelial cell showing obvious pathological features like in fig. 5 but much more extensive. Note the adjacent cell (lower right corner) of the same section looking normal. Symbols as in fig. 4, and: RE, rough endoplasmic reticulum; LY, lysome-like structures.

altered or apparently associated with lysosome-like structures which have become remarkably numerous; ribosomes strongly reduced in numbers and rough endoplasmic reticulum almost disappeared; apical cytoplasm with the above mentioned vacuoles further enlarged and merging with each other to form a sort of extended sponge (fig. 7b) which eventually leads to the cell rupture; basal labyrinth completely disorganized (fig. 7c). While the midgut muscular sheath (figs 7c and 8) as well as its associated connective tissue (fig. 7c) appear almost completely disrupted.

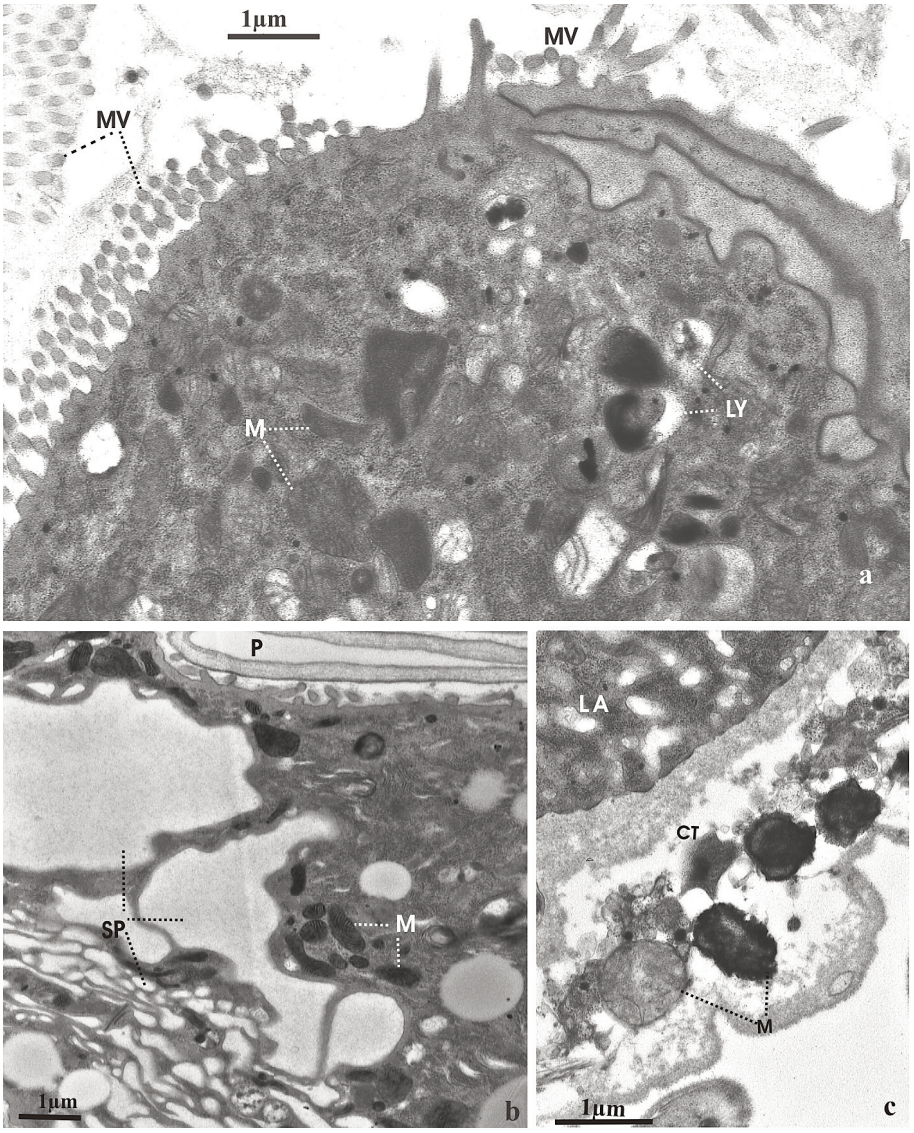

Fig. 7 - *Musca domestica* adult female sacrificed 72 hrs after emergence and being fed on spore suspension of *Bacillus* sp. new isolate. T.E.M. micrographs from midgut epithelial cell section details showing: a, dramatic deterioration of the whole cytoplasm, especially microvilli; b, apical aspect of a nearly ruptured cell, still displaying pathological features at maximum extent; c, basal aspect of the same cell showing strongly altered labyrinth; while muscular sheath and connective tissue appear completely disrupted. Symbols as in figs. 4, 5 and 6, and: SP, spongy aspect of a very apical portion (towards gut lumen) of a rupturing cell.

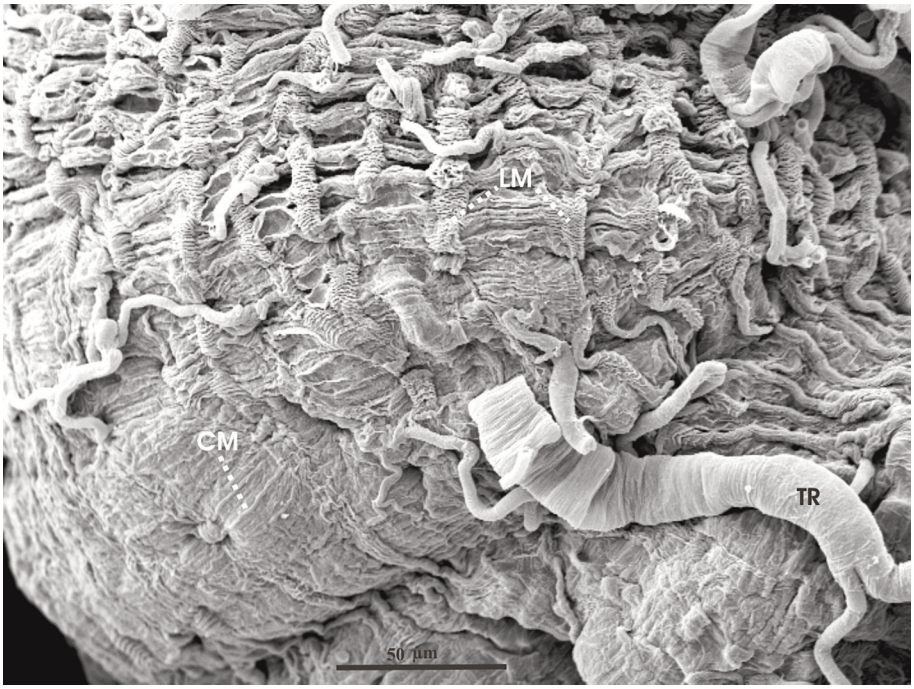

Fig. 8 - *Musca domestica* adult female sacrificed 72 hrs after emergence and being fed on spore suspension of *Bacillus* sp. new isolate. S.E.M. micrograph of midgut detail, close to pyloric valve, displaying muscular sheath strongly deteriorated, especially longitudinal fibrils. CM, circular muscular fibrils; LM, longitudinal muscular fibrils; TR, tracheae.

In the samples with flies sacrificed 48 hrs after emergence and being fed on the culture supernatant of *Bacillus* sp., intoxicated midgut epithelial cells display pathological features similar to those on flies treated with the spore suspension of the same *Bacillus* sp. (compare fig. 9 with figs. 6 and 7) although as a whole at lower degree. These effects could be due either to the presence of spores (as confirmed by phase microscope observations of the culture supernatant) or some other components of the sporulated culture at a very low concentration, or to some soluble compounds with a weak toxicity.

In the sample with flies sacrificed 48 hrs after emergence and being fed on a spore-crystal suspension of *B. th*. var. *kurstaki*, very few histopathological alterations on the midgut epithelial cells can be detected (there is only an apparent microvilli deterioration on the intoxicated cells: fig. 10a), as attended by the normal behaviour and low mortality rate displayed by this fly sample (see above).

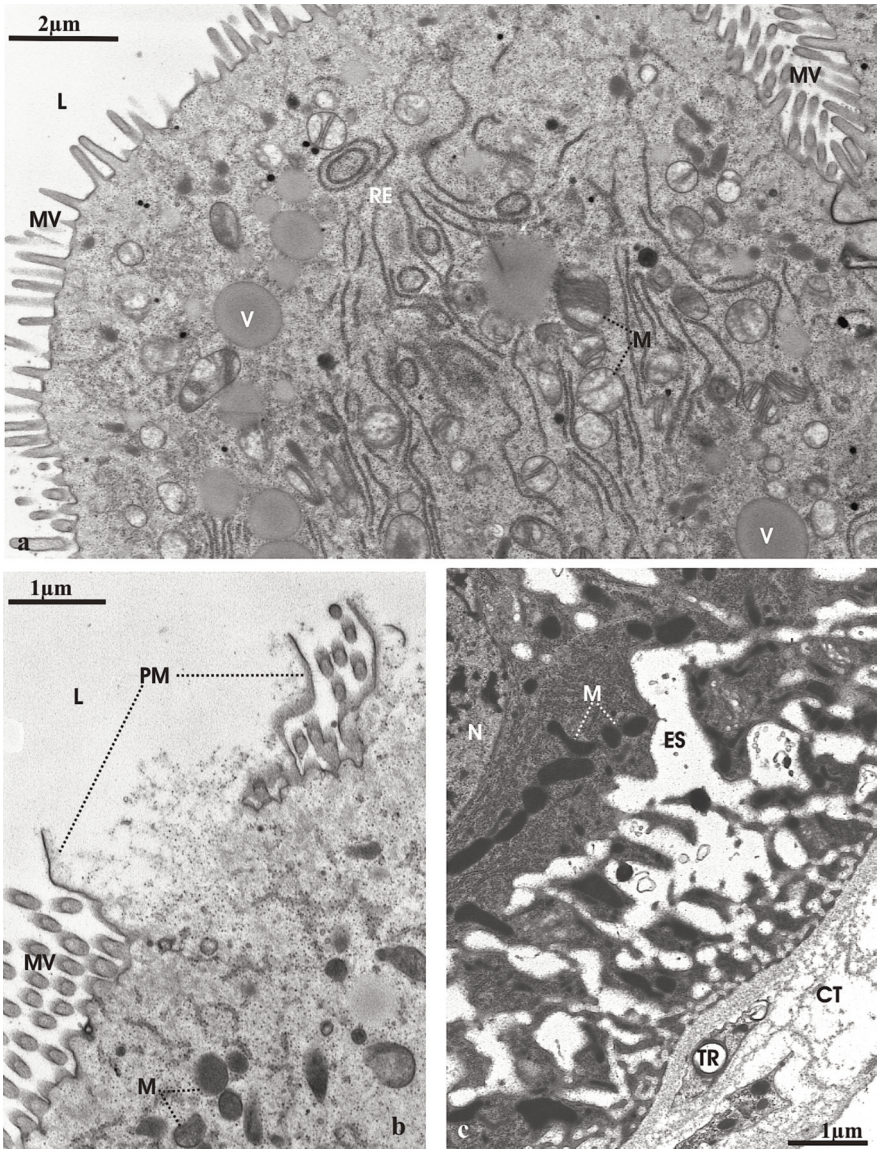

Fig. 9 - *Musca domestica* adult female. Same sample as in fig. 3c, i.e., specimen sacrificed 48 hrs after emergence and being fed on *Bacillus* sp. culture supernatant. T.E.M. micrographs from section details of midgut, showing: a, epithelial cell apical region with extensive degeneration of microvilli, many mitochondria obviously swollen and disrupted, rough endoplasmic reticulum disorganized; b, cell membrane ruptured (left upper corner) and allowing cytoplasm to leak into the gut lumen; c, epithelial cell basal region with labyrinth strongly deteriorated and displaying wide extracellular spaces. Symbols as in fig. 4, and: RE, rough endoplasmic reticulum; ES, extracellular space; PM, cell membrane; V, vacuoles.

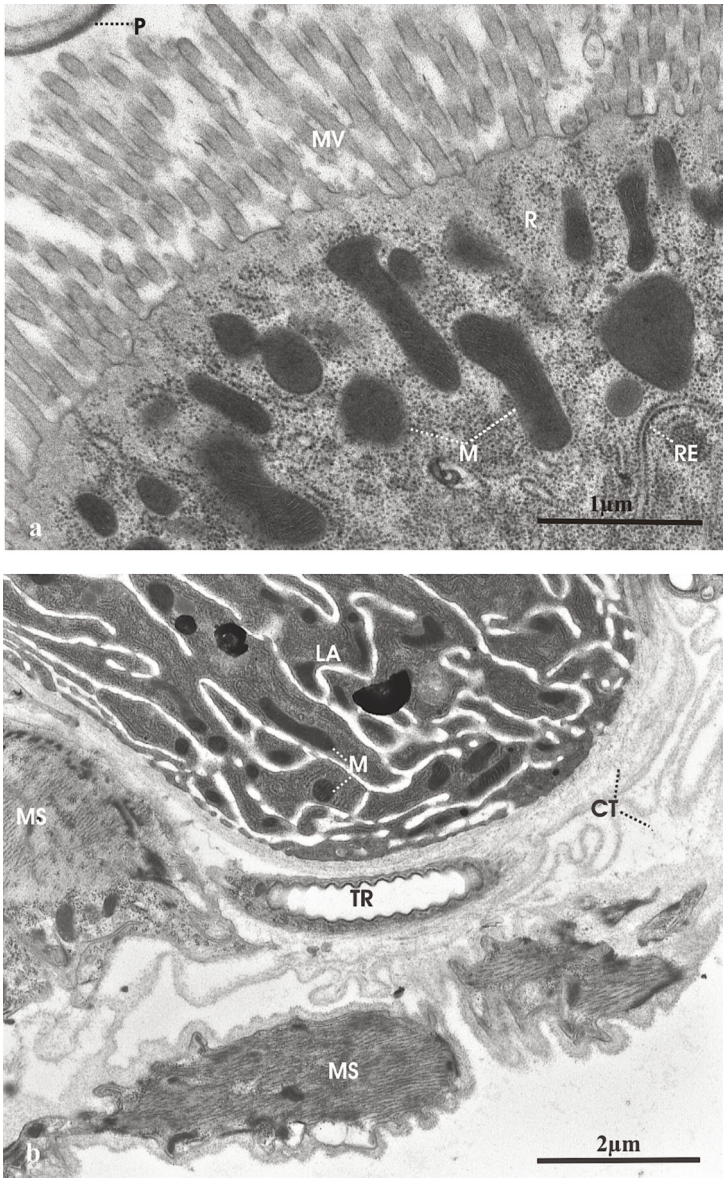

Fig. 10 - *Musca domestica* adult female sacrificed 48 hrs after emergence and being fed on spore-crystal suspension of *B. th.* var. *kurstaki* (HD-1). T.E.M. micrographs from section details of midgut: a, epithelial cell showing apical region with deteriorated microvilli; b, the same cell basal region and muscular sheath looking almost normal (compare Fig. 4, control). Symbols as in Fig. 4, and: MS, muscular sheath; R, ribosomes.

The malpighian tubule upper segment of flies sacrificed 48 hrs after emergence and being fed on the spore suspension of *Bacillus* sp., display epithelial cells with pathological features similar (fig. 12b) to those above described for intoxicated midgut epithelial cells, up to cell rupture.

4. DISCUSSION

Musca domestica adults treated with a spore suspension of *Bacillus* sp. display progressive pathological effects such as sluggish behaviour and general paralysis up to death, as reported for, e.g., *Bombyx mori* larvae (ENDO and NISHIITSUTSUJI-UWO, 1980) treated with *B. th.* var. *kurstaki* and *aizawai*, or *Periplaneta americana* adults (SINGH and GILL, 1985) and *M. domestica* larvae (SINGH *et al.*, 1986) treated with *B. th.* var. *israelensis*. The latter both interpreted as due to a general myotoxic and neurotoxic activity of δ-endotoxins released by *B. th.* var. *israelensis*.

Histological and ultrastructural investigations on flies treated with the spore suspension of *Bacillus* sp. evidence midgut and malpighian tubules' damages (such as: progressive deterioration of epithelial cell cytoplasm, essentially consisting of microvillar disruption, endoplasmic reticulum vacuolation, mitochondrial degeneration, excessive numbers of lysosome-like structures, loss of ribosomes and rough endoplasmic reticulum disorganization, disappearance of basal infoldings, up to cell rupture; as well as deterioration of midgut muscular sheath and related connective tissue) very similar to those reported for other insects from different taxonomic groups treated with δ-endotoxins from different strains of *B. thuringiensis*.

In fact, *M. domestica* adults fed on spore suspension of *Bacillus* sp. display midgut epithelial cells having progressive: a) endoplasmic reticulum vacuolation like, e.g., for *B. mori* larvae treated with *B. th*. var. *kurstaki* or *aizawai* (ENDO and NISHIITSUTSUJI-UWO, 1980; PERCY and FAST, 1983), for *Manduca sexta* larvae treated with *B. th*. var. *kurstaki* (LANE *et al.*, 1989), for *Chrysomela scripta* F. larvae treated with *B. th*. var. *san diego* (BAUER and PANKRATZ,1992), and for *Dociostaurus maroccanus* treated with *B. th*. var. *aizawi* (QUESADA-MORAGA and SANTIAGO-ALVAREZ, 2001); b) microvillar disruption as reported, e.g., for larval midgut of *Bombyx mori* (PERCY and FAST, 1983) or *Manduca sexta* (LANE *et al.*, 1989), both treated with *B. th*. var. *kurstaki*; c) mitochondrial degeneration like, e.g., for *B. mori* larvae treated with *B. th*. var. *kurstaki* or *aizawai* (ENDO and NISHIITSUTSUJI-UWO, 1980); d) excessive numbers of lysosome-like structures like, e.g., for *M. sexta* larvae treated with *B. th*. var. *kurstaki* (LANE and LEE, 1989), and for *Ch. scripta* larvae treated with *B. th*. var. *san diego* (BAUER and PANKRATZ, 1992); e) loss of ribosomes and rough endoplasmic reticulum disorganization like, e.g., for *Ch. scripta* larvae treated with *B. th*. var. *san diego* (BAUER and PANKRATZ, 1992), and for *B. mori* larvae treated with *B. th*. var. *kurstaki* (PERCY

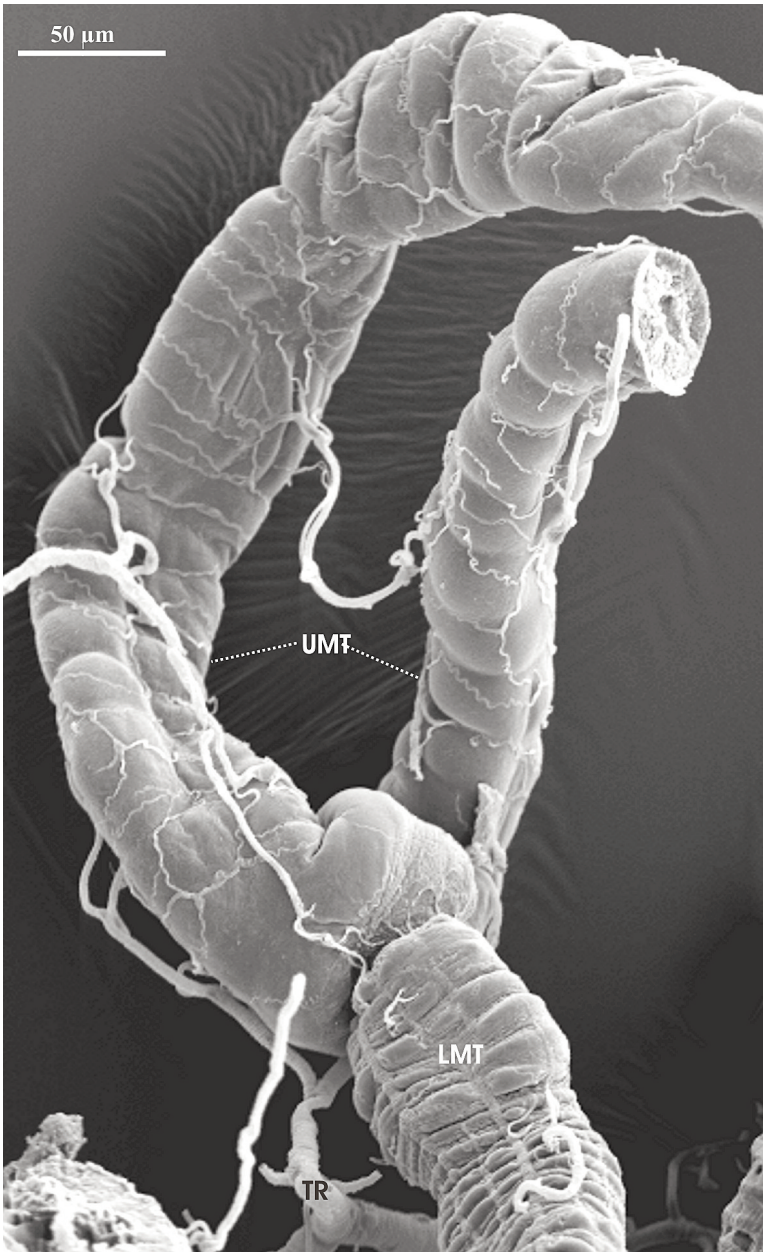

Fig. 11 - *Musca domestica* adult healthy female 48 hrs old. S.E.M. micrograph displaying malpighian tubule detail. LMT, malpighian tubule lower segment (ureter); TR, tracheae; UMT, malpighian tubule upper segments.

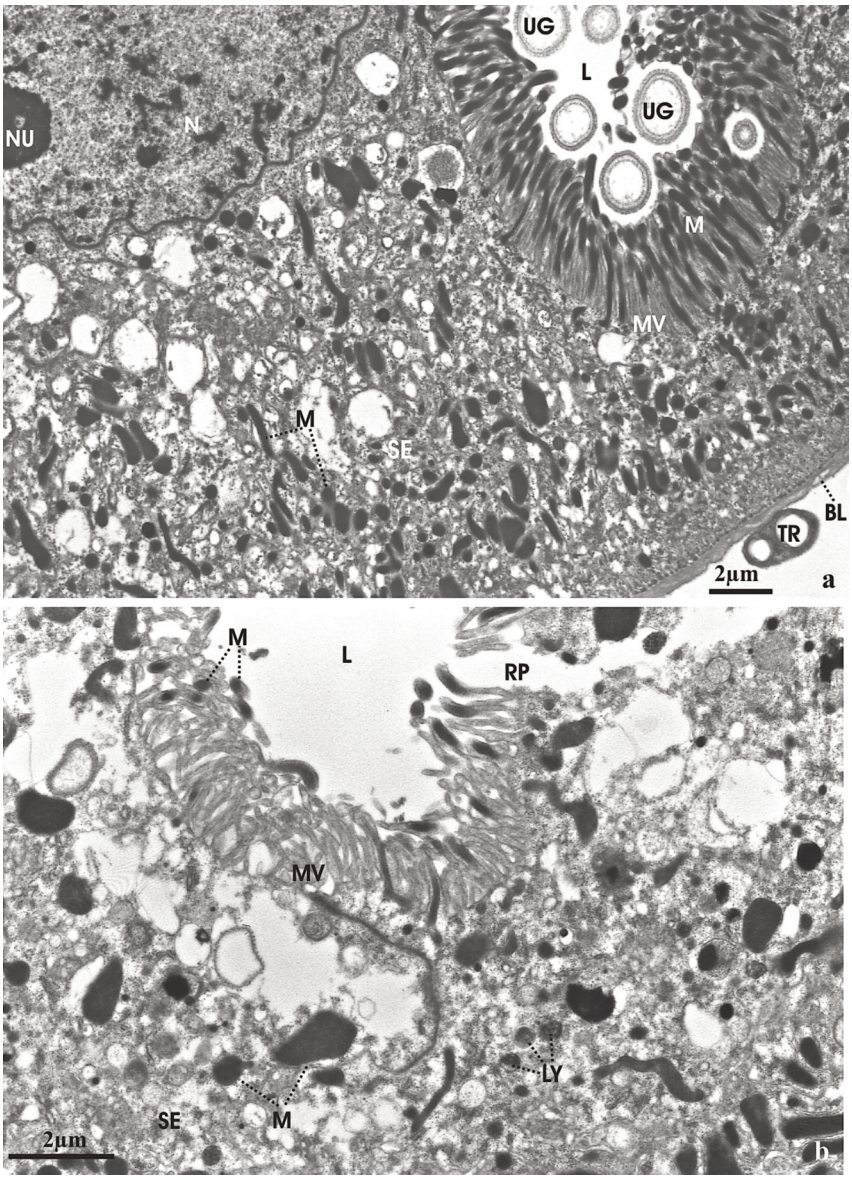

Fig. 12 - T.E.M. micrographs from section details of malpighian tubule upper segment of *Musca domestica* adult female: a, healthy specimen 48 hrs old; b, specimen sacrificed 48 hrs after emergence and being fed on the spore suspension of *Bacillus* sp. new isolate. Note epithelial cell pathological features similar to intoxicated midgut epithelial cells. Symbols as in fig. 9, and: BL, basal lamina; L, malpighian tubule lumen; UG, urate granules.

and FAST, 1983); f) disappearance of basal infoldings like, e.g., for *B. mori* larvae treated with *B. th*. var. *kurstaki* and *aizawai* (ENDO and NISHIITSUTSUJI-UWO, 1980); g) deterioration of midgut muscular sheath and related connective tissue like that reported for the body-wall muscles of *M. domestica* larvae treated in vitro with δ-endotoxin from *B. th.* var. *israelensis* (SINGH *et al*., 1986).

It is remarkable that, analogously to what reported for other insect species treated with different varieties of *B. th*. δ-endotoxins, the mentioned intoxication effects produced by *Bacillus* sp. do not affect the whole midgut epithelium, but only selected cells, i.e., those presumably provided with specific membrane receptors (QUESADA-MORAGA and SANTIAGO-ALVAREZ, 2001); whereas the midgut muscular sheath appears almost always severely damaged, maybe due to a general susceptibility to endotoxin intoxication of the whole muscular system (SINGH and GILL, 1985; SINGH *et al*., 1986).

In the flies treated with culture supernatant of *Bacillus* sp., intoxicated midgut epithelial cells display pathological features similar to those in flies treated with the spore suspension of *Bacillus* sp. although as a whole at lower degree, like reported for *Culex siriens* larvae treated with exotoxins from *B. th*. H-1 Berliner (WEISER and ZIZKA, 1994) and for *Bovicola ovis* treated with both spore-crystal mixture and culture supernatant of the same *B. th*. strain (HILL and PINNOCK, 1998). In our case, however, the results are not directly comparable to the above mentioned reports because the supernatant was not sterilised and contained some spores. Moreover, no significant symptoms of intoxication were detected on flies behaviour assays.

The flies treated with spore-crystal suspension of *B. th*. var. *kurstaki*, evidence very weak histopathological alterations on the midgut epithelial cells, as to confirm the normal behaviour and very low mortality rate displayed by this fly sample.

The malpighian tubules of flies treated with spore suspension of *Bacillus* sp., display epithelial cells with pathological features similar to those of intoxicated midgut epithelial cells, as reported for the malpighian tubules of *Rhodnius prolixus* treated with δ-endotoxin from *B. th*. var. *israelensis* (MADDRELL *et al*., 1988).

5. CONCLUSIONS

From the above results and discussion, the following essential conclusions may be drawn.

A spore suspension of a new isolate of *Bacillus* sp., close related to *B. thuringiensis* proved considerable insecticidal activity on *M. domestica* adults in laboratory behavioural bioassays, as well as through anatomo-histological and ultrastructural investigations.

In fact:

Behavioural bioassays evidence that flies treated with the spore suspension of *Bacillus*

sp., after 48 hrs, show sluggish and shaky behaviour, decreased responsiveness to external stimuli and gradual feeding inhibition, while mortality already appears significantly higher than in control flies; and it gets over 52%, 72 hrs after treatment, when most of remaining specimens undergo general paralysis, until almost all treated flies gradually die within 1-2 days later on; whereas survived control flies still look normal.

Comparative anatomo-histological and ultrastructural investigations clearly confirm the above results from behavioural bioassays, proving that flies treated with *Bacillus* sp. undergo progressive degeneration (up to rupture) of midgut and malpighian tubules' epithelial cells, as well as of the midgut muscular sheath, eventually causing lethal effects on flies, such as most of those reported for other insects treated with *B. thuringiensis*.

Parallel tests on flies treated with analogous suspension of *B. th*. var. *kurstaki* (HD-1) gave rather negative results.

Finally, intoxicated midgut cells of flies fed with the culture supernatant, compared to those of flies fed with the spore suspension of *Bacillus* sp., displayed similar alterations, although at a quite lower degree, but without significant detectable changes in flies behaviour or viability. It clearly suggests that the major toxic component is contained in the spore suspension obtained harvesting and washing the whole sporulated culture.

Moreover, the affinity of the observed midgut alterations caused by the new bacterial isolate on adult house fly with those caused by different *B. thuringiensis* enthomopathogenic toxins on various insect pests, suggest it could be due to similar mechanism of action. Neverthless, more study is in progress to identify the responsible toxic factors.

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RIASSUNTO

RICERCHE COMPORTAMENTALI ED ANATOMO-PATOLOGICHE SU ADULTI DI *MUSCA DOMESTICA* L. TRATTATI CON UN NUOVO ISOLATO DI *BACILLUS* SP. AFFINE AL *B. THURINGIENSIS* (BERLINER).

Biosaggi comportamentali e indagini anatomo-patologiche sono state parallelamente condotte su adulti di *Musca domestica* L. per provare l'attività insetticida di un nuovo isolato di *Bacillus* sp. (attualmente in via di caratterizzazione), affine al rinomato *Bacillus thuringiensis* (Berliner). Sia le prove comportamentali che quelle anatomo-istologiche ed ultrastrutturistiche hanno dato risultati simili a quelli riportati in letteratura per vari altri insetti trattati analogamente con diverse varietà di *B. thuringiensis*. Mosche alimentate con una sospensione della coltura sporulata di *Bacillus* sp. vanno incontro progressivamente a intorpidimento generale e tremore, ridotta sensibilità a stimoli esterni, graduale rifiuto del cibo e paralisi generale, fino alla morte (oltre 52% delle mosche muoiono entro 72 ore, e quasi tutte in 4-5 giorni dalla prima somministrazione della sospensione batterica). Le osservazioni anatomo-istologiche ed ultrastrutturistiche corroborano i risultati dei biosaggi comportamentali, evidenziando nelle mosche trattate col nuovo isolato di *Bacillus* sp. una progressiva degenerazione delle cellule epiteliali del mesentero e dei tubi malpighiani, come pure della tunica muscolare del mesentero, come è stato visto per altri insetti trattati con *B. thuringiensis* (Berliner). Prove di confronto con adulti di *M. domestica* analogamente trattati con una sospensione di *B. th* var. *kurstaki* (HD-1) hanno dato risultati sostanzialmente negativi.

Parole chiave: anatomia intestinale, attività insetticida, biosaggi comportamentali, istologia mesenteriale, microscopia elettronica, microscopia ottica.

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