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DD Barsagade
Department of Zoology, MJF Educational Campus, RTM Nagpur University, Nagpur, Maharashtra, India.

HM Meshram
Department of Zoology, MJF Educational Campus, RTM Nagpur University, Nagpur, Maharashtra, India.

Correspondence
HM Meshram
Department of Zoology, MJF Educational Campus, RTM Nagpur University, Nagpur, Maharashtra, India.

Impact of *Bacillus subtilis* on midgut and silkgland of tropical tasar silkworm, *Antheraea mylitta* (D) Eco-race, Bhandara

DD Barsagade and HM Meshram

Abstract
Tropical tasar silkworm *Antheraea mylitta* is economically important insect and it produced famous tasar silk commonly known as 'Kosa Silk'. The Indian tropical tasar silkworm is affected by disease flacherie (bacteriosis) caused by bacteria, which accounts considerable loss of 10-15 % silk cocoon production. In the present investigation histopathological effect of diseases causing bacteria in tasar silkworm, *Antheraea mylitta* has been studied. The bacteria from diseased rectal protrusion were isolated on specific growth media, identified as *Bacillus subtilis* and its effects on midgut and silk gland of tasar silkworm were investigated. During early infection, midgut epithelial cell size increased with vacuole formation. During late stage of infection, mid gut columnar epithelium cells showed hypertrophy and blabbing of plasma membrane, disruption of microvilli, vacuolization and permanent foremost nuclear changes to necrosis. The silk gland of diseased larvae showed cyto-morphological changes with reduced cell size and vacuole formation in early infection. The progressive disintegration of cytoplasmic organelles occurs with advancement of diseased in silk gland.

Keywords: *Antheraea mylitta*, eco-race, histopathology, midgut, fat body and silk gland

1. Introduction
The major factor responsible for outbreak of flacherie (bacterial disease) is found to be change in environmental condition mostly, variation in temperature, rainfall and humidity percentage. The bacteriosis leads to dysfunction of alimentary canal, and encourages outbreak of flacherie [23]. The bacteriosis caused by different types of bacteria and prevail all throughout the year in tasar culture region. The impact is more pronounced in the larval stages which affect cocoon productivity and quality along with 20-25% crop loss [31]. The bacteria that induced flacherie includes, *Bacillus* spp., *Streptococcus* spp., *Staphylococcus* spp., *Bacillus thuringiensis*, *Serratia marcescens*, *Micrococcus* spp. *Proteus* spp., *Pseudomonas* spp., and *Aerobacter cloacae* [26, 36]. An extensive study has been carried out on the bacterial infection in relation to mortality of larvae, pathogenic effect and characterization of bacteria in *B. mori* [1, 32]. There is very meager information on the bacterial pathogenic effect and larval mortality in *Antheraea mylitta* except few reports [20]. The pathogenic effect of bacteria *Bacillus subtilis* on the various vital organs were investigated in the present study.

2. Materials and methods
The rearing of tasar silkworm, *Antheraea mylitta* eco-race Bhandara for several generation were carried out in rearing field at Gadchiroli and Chandrapur district of Maharashtra during month of July to January in the year 2010-2013.

2.1 Isolation and purification of bacteria
The fifth instar larvae of tropical tasar silkworm, *A. mylitta* (D) of I, II and III cycle showing the symptoms of flacherie were brought from the field (Fig.1 and 2). The infected larval cadavers were homogenized in distilled water and filter it. The filtrate was inoculated in a nutrient agar medium for 24 hrs. After growth of bacterial colony, a single colony of bacteria was picked up and streaked on specific growth media. The bacterial culture was identified on the basis of growth on specific media, colour of bacteria colonies, morphological characteristic and gram staining reactions. The specific growth media were used *i.e*. High chrome *Bacillus* Agar (HBA) for gram positive bacteria *Bacillus* spp.

For the isolation of pure strain of bacteria, *Bacillus sp.* colony was cultured on High chrome *Bacillus* Agar specific growth medium and *B. subtilis* formed green colony on HB (Fig.3 and 4). The pure strains of *B. subtilis* were stored at 4ºC temperature for further laboratory used.

Fig 1: Healthy fifth instar larva of tasar silkworm *A. mylitta*

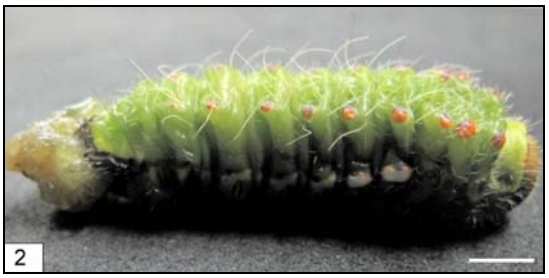

Fig 2: Bacterial infected fifth instar larva of tasar silkworm *A. mylitta* showing sealing of anal lips (arrow)

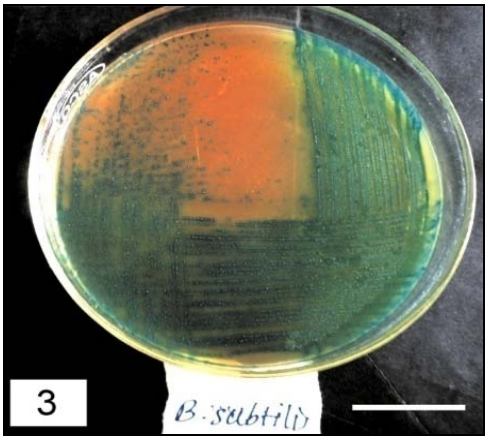

Fig 3: Culture of bacterial strain grown in the specific High chrome *Bacillus* agar culture medium showing green colony of *Bacillus subtilis*

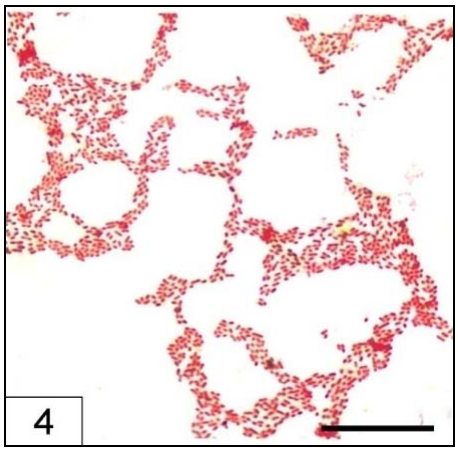

Fig 4: Gram +ve bacteria, *Bacillus subtilis*. Gram staining (X 400)

2.2 Histopathological study

The larvae from healthy and diseased group were dissected and the tissues such as midgut and silk gland were fixed and embedded in paraffin wax for histopathological study. Sections of healthy and infected tissues were stained with haematoxylin eosin (HE) [19].

The cell and nucleus size were calculated and probability of significant (P- value) were calculated from the Fisher's table of 't' value [34].

3. Result

3.1 Histopathological changes in the midgut, and silk gland

The infected larvae were categorized into early and late infected larvae on the basis of infectious symptoms such as sealing of anal lips. These larvae were dissected gently and mid gut, fat body and silk glands were separated. The dissected tissues were preceded to microtomy preparation and histopathological changes in the midgut, fat body and silk glands were observed (Table 1). Similarly normal healthy larvae from same field were used for control study.

3.2 Midgut (Control)

In the healthy fifth instar larvae of *A. mylitta* (D), the midgut represents the largest part of the alimentary canal and the wall of the midgut is composed of outer muscle layer and inner epithelial layer. The midgut epithelium is greatly folded and rests upon a basement membrane. The cells of midgut epithelium are differentiated into three types, the columnar, the goblet and the regenerative cells).Columnar cells are tall and closely associated with each other so that their boundaries are indistinct. They are interspersed apically with goblet and basally with regenerative cells. These columnar cells contain granular cytoplasm. The nuclei are large, spherical or elliptical and situated in the middle or apical half of the cells. The Goblet cells are large flask or vase shaped, modified epithelial cells. The goblet cells contain centrally the oval nuclei and bulk of cytoplasm in the basal region. The regenerative cells are small and irregular shaped in the larvae. (Fig. 5).

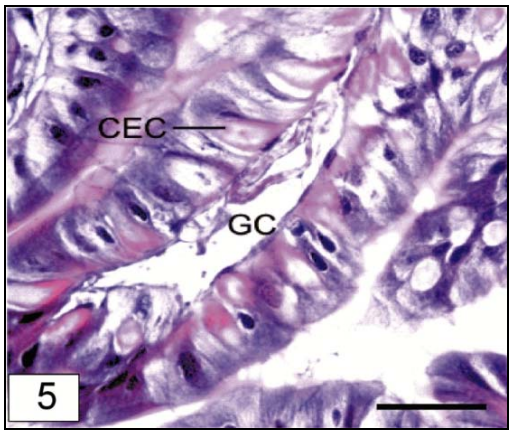

Fig 5: Cross section of mid gut of healthy fifth instar larva of tasar silkworm *A. mylitta* showing columnar epithelial cell and compact darkly stained nucleus, goblet cell, muscle layer and regenerative cell (Bar = 4.2 µm).

3.3 Midgut (Early Infected)

During the early stage of infection, the epithelial layer thickness and width increased than the controls. The columnar cells increased in shape and size with formation of vacuoles at the central and basal regions of the cells (Table- 1). The

nuclei migrated towards the basal region of the cells and appear globular in shape. The length and width of the nucleus decreased significantly than the healthy midgut cells. The chromatin material was visible in nuclei. The brush border lining of the cells toward the lumen were intact, but at few places, it was broken down. The basement membrane and muscle layer were attached with the columnar epithelial cells. At few places, detachment of the muscle layer was observed. Some cells were small sized with centrally placed nuclei. The vacuoles were distributed throughout the cells (Fig. 6).

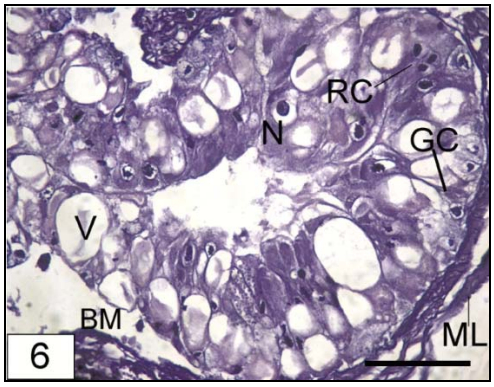

Fig 6: Cross section of mid gut after early stage of bacterial infection showing vacuoles in columnar epithelial cell, goblet cell and regenerative cell, brush border membrane towards lumen and vacuoles in cytoplasm (Bar = 4.2 µm).

3.4 Midgut (Late Infected)

During the late stage of infection, the columnar cell length and width showing significant reduction as compared to that in the healthy (Table 1). The cytoplasmic granular inclusions were degenerated and cells become completely empty. The cell membrane was not affected by bacteria, and found intact. The nuclei were escaped from the cells or completely disappeared. It was difficult to identify the nuclei in the cell while necrosis of the cells and nuclei were observed. The brush border membrane was completely degenerated. The muscle layers were completely detached and separated from the epithelial layer (Fig. 7).

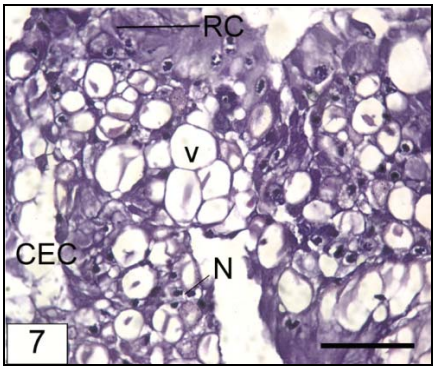

Fig 7: Cross section of mid gut after late stage of bacterial infection showing hypertrophied columnar epithelial cell, goblet cell and regenerative cell and vacuoles in cytoplasm. (Bar = 4.2 µm).

Table 1: Effects of *Bacillus subtilis* on midgut columnar epithelial cells of the fifth instar larva of *A. mylitta* (D) after early and late stage of infection

Name of tissue	Columnar epithelial cells		Nucleus		Epithelial cell thickness(10x)µm
	Length (µm)	Width (µm)	Length (µm)	Width (µm)	(10x)µm
Healthy	35.0 ± 4.02	22.0 ±3.02	18.9 ± 4.7	12.3 ± 2.8	57.4 ± 9.6
Early stage	41.0* ±5.02	26.3* ± 3.6	10.1 ± 2.2	8.2 ± 0.1	58.03*** ± 7.3
Late stage	22.9 ± 4. 6	14.8 ± 2.2	**	**	27.9 ± 3.2

± Mean standard error. ** complete destruction, *** P > 0.01, * P >0.05, P<0.05

3.5 Silk Gland (Control)

In the freshly dissected larvae the middle milk gland was milky white and differentiated predominantly in to anterior, middle and posterior regions of silk gland. The silk glands were made up from single layer of epithelial cells. The cells were elliptical, oblong and flask shaped. The nuclei were large initially, but become horse-shoe shaped and elongated during secretary activity. The chromatin material was fine granular and dispersed throughout the nuclei but sometimes accumulated at the center. The lumen was filled with variable amount of secretary materials (Fig. 8).

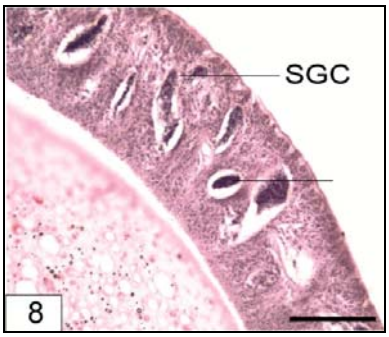

Fig 8: Section passing through the posterior silk gland (PSG) of healthy fifth instar larva of *A. mylitta,* showing horseshoe shaped nuclei and silk protein secretion in lumen (Bar = 3.5 µm).

3.6 Silk Gland (Early Infected)

During early stage of infection of bacteria, in the fifth instar larvae the diameter of silk gland show decrease significantly as compared to that in the healthy. The thickness of silk gland wall was also reduced. The nuclear size was changed and became small, spherical, elongated or horseshoe shaped showing various deformities. The sizes of nuclei were reduced significantly (Table - 2). The nuclei were filled with granular chromatin materials and few vacuoles were observed in the cytoplasm of epithelial cells (Fig.9).

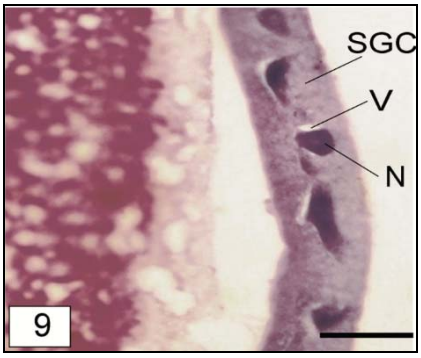

Fig 9: Section passing through PSG in early stage bacterial infected larvae of *A. mylitta* showing, small spherical nuclei and appearance of vacuoles in silk gland cell (Bar = 3.5 µm).

Table 2: Effects of bacteria, *Bacillus subtilis* on silk gland of the fifth instar larva of*A. mylitta* (D) after early and late stage of infection

Name of tissue	Diameter of silk gland (10x) (µm)	Lumen diameter (10x)(µm)	Thickness of Epithelium (µm)	Nucleus	
				Length(µm)	Width(µm)
Healthy	1.2 ± 0.2	1.2 ± 0.1	114.8± 18.4	42.6 ± 5.5	18.1 ± 4.6
Early stage	1.1 ± 0.2	1.1 ± 0.2	58.1 ± 7.3	26.2 ± 2.2	13.9 ± 2.2
Late stage	0.7 ± 0.1	0.5 ± 0.1	22.9 ± 3.2	15.5 ± 2.1	2.1 ± 0.7

± mean standard error, P< 0.05

3.7 Silk Gland (Late Infected)

During late stage of infection, silk gland diameter significantly decreased as compared to healthy larval silk gland. The thickness of silk gland epithelial cell wall was reduced. The nuclear size reduced and became elongated shaped (Table -2). The chromatin material was very compact. The cytoplasm become scanty and some empty vacuoles were observed in cytoplasm. Negligible amount of secretary material was observed in the lumen (Fig. 10).

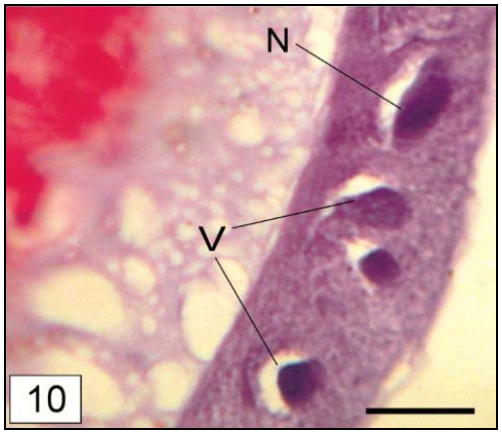

Fig 10: Section passing through PSG in late stage bacterial infected larvae of *A. mylitta* showing thin elongated darkly stained nuclei and vacuoles appeared in the cytoplasm (Bar = 3.5 µm).

4. Discussion

Pasture (1870), has reported the bacteria as the etiological agents of flacherie in the silkworms. In silkworm, *Bombyx mori* various species of *Bacilli* have been reported as *Bacillus megaterium, Bacillus bombyseticus* [16], *Bacillus bombycoides* [27] and *Bacillus mycoides, Bacillus laterosporus* [35]. During the present study gram positive bacteria *Staphylococcus aureus, Bacillus cereus* and *Bacillus subtilis* has been identified among them isolated *B. subtilis* found as a major pathogen during 1st, 2nd and 3rd life cycle in a year [15]. The isolation of *B. subtilis* from the cadavers of *A. mylitta* eco-race Bhandara is first report and it confirms that *B. subtilis* are major pathogenic bacteria causing flacherie to the tasar silkworms. Bacterial pathogen such as *Bacillus* spp. and *Staphylococcus aureus* were identified in *B. mori* [1]. The occurrence of *Staphylococcus* in silkworm has been reported [28]. Similarly, there are few reports on occurrence of gram negative bacteria, *Pseudomonas aeruginosa* in silkworms [3]. The *Bacillus* sp. was found distinctly in *A. mylitta* during the present observations. Perhaps this is the first report on *B. subtilis* causing flacherie *in A mylitta.*

Earlier identified several species of bacteria as insect pathogen like *Bacillus thuringiensis, Serratia marcescens, Pseudomonas aeruginosa* and *Proteus vulgaris* [6]. During present study three species of bacteria *i.e. Staphylococcus aureus, Bacillus cereus* and *Bacillus subtilis* has been identified as pathogens of *A. mylitta*, among them *B. subtilis* found as a major pathogen during 1st, 2nd and 3rd life cycle (crop) of *A. mylitta* in a year. Loss of weight and diet of silkworm is common characters during flacherie. It has been found that after infection of *Bacillus thuringiensis* infected larvae stop feeding and weight of wax moth larvae decreased down two times within period of 3 days [12]. It is well known that during bacteriosis the initial acute stage of diseases is the effect of toxin [5, 12].

In the present study it has been found that after *B. subtilis* infection, larvae become sluggish, weak stop feeding and ultimately died. Increased larval mortality during *B. subtilis* infection may be due to increased concentration of endotoxin in the midgut of infected tasar silkworm larvae.

Histopathological studies on mode of action of bacterial toxin on the midgut epithelium of larvae of silkworm, *B. mori* [29], *P. brassicae* [21] and tobacco hornworm, *M. sexta* [17] has been studied earlier. The mechanism of action of toxin involves activity against the epithelium of midgut. After the ingestion of pathogenic material by the susceptible insect, the insoluble bipyramidal crystalline toxin is solubilized in gut lumen, under the action of high alkaline pH and intestinal proteases [14, 25]. As a result, these protoxins are converted to the active toxins and bind to the specific receptors present in the brush border membrane vesicles (BBMV) of the columnar cells [18]. The binding is however, followed by insertion of the toxins into the apical microvilli membrane of columnar epithelial cells [2, 30]. The interaction of toxin with those receptors which have been characterized as aminopeptidase-N, trigger and formation of ionic channels (pore formation) disrupting the osmotic-equilibrium and permit an inflow of ions and water thereby modifying the intracellular cation levels leading to the cell swelling [4, 11]. The sequential changes in structure of midgut epithelium were found such as irregular swelling and distortion of microvilli of columnar cells and goblet cell. The formation of large vacuoles was found in cytoplasm of columnar and goblet cells in *B. mori* and *P. brassicae* [29]. The nuclei of these cells are found displaced by karyolysis and pycnosis [7]. During present study, swelling of columnar cells, damaged microvilli or brush border membrane and formation of large cytoplasmic vacuoles were observed. The displacement of nuclei toward the basal region is observed during the early infection stage of *B. subtilis* to the *A. mylitta* (D).

In *B. mori* and *P. brassicae* larvae, the delta- endotoxin caused volume increase of gut epithelial cells. Later on, the columnar and goblet cells lose their structural organization. The cytoplasmic vacuolization and nuclei appeared abnormally condensed. These all cellular lesions are assumed to be crucial to the gut paralysis [9]. In *A. mylitta* after late stage of *B. subtilis* infection, epithelium show drastic changes and nuclei of affected cells disintegrate completely. Accordingly the cells swell and ultimately burst, the process known as colloid-osmotic lysis [9, 13]. This leads to disruptions of gut integrity, finally intoxicated *A. mylitta* stops feeding and ultimately death of *A. mylitta* results due to anorexia. The death of insect due to anorexia or cessation of feeding or starvation or septicemia in bacterial infected insect is very common. The present study confirmed disruption of gut lead

to death of *A. mylitta* after infection of *B. subtilis* [10].

The silk gland also become an ideal ground for the growth of pathogenic bacteria [22]. Bacteria in the silkworm larvae mostly infect silk gland through the digestive tract and less commonly through the egg, integument and trachea. The chain forming bacilli and subsequently occurrences of lysosome bodies in posterior silk gland cell of *A. mylitta*, suggesting the antibacterial mechanism in posterior silk gland after bacteriosis [37]

The multiplication of flacherie infected bacterium leads to poor intake of food that are occupied in the larval gut lumen and histopathological changes in gut epithelium had affected the energy budget in the silkworms [33]. The poor supply of energy, damage the silk protein in the silk gland of silkworm. The pathogenic microbes had caused a breakdown in protein synthetic machinery in the silk gland [8]. In the study confirmed that bacteria secondarily infected the silk glands. During the infection, silk gland diameter significantly decreases as compared to that in the controls. The thickness of silk gland epithelial cell wall reduced. The nuclear size becomes small with the empty vacuoles in cytoplasm and the cytoplasm become scanty. Negligible amount of secretary material is observed in to lumen.

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