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Alteration of gut histology and induced toxicity in flacherie infected muga silkworm, *Antheraea assamensis* Helfer (Saturniidae: Lepidoptera)

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Abstract
Antheraea assamensis Helfer, known as muga silkworm being wild in nature are exposed to various conditions of changing environment. Therefore, the muga silkworms are prone to various bacterial diseases including flacherie. In present study microbial strains *Staphylococcus aureus* strain FLG1 (KR025521), *Bacillus thuringiensis* strain MK1 (KR069143) and *Pseudomonas aeruginosa* strain DRK1 (KP688076) isolated from the gut of flacherie diseased muga silkworm were used to study their effect on alanineaminotransferase (ALT) activity and gut histology. Study results revealed that both oral administration and injected groups of larvae had an altered activity of ALT in different time interval. The midgut of oral administration group showed higher enzymatic activity than foregut and hindgut, however infected groups showed higher activity than control one. At 24 h, both *B. thuringienesis* and *P. aerogonisa* showed significantly higher ALT activity, however in *S. aureus* infected group the alteration was not significant at 48 and 72 h. Moreover, in bacteria injected group, 24 h of infection did not show significant alteration in foregut, however at 48 and 72 h significantly higher ALT activity was observed. The midgut and hindgut showed significantly higher ALT activity at 24 – 72 h of infection period. Similarly in oral administered groups, alteration in gut line, degenerative changes in mucous layer, broken and fused microvilli were observed.

Keywords: Muga silkworm, flacherie, alt activity, gut histology

1. Introduction
Antheraea assamensis Helfer, known as muga silkworm is an economically important insect species endemic to the North Eastern region of India. It occurs in the Brahmputra valley in Assam, East, West and South Garo hills of Meghalaya, Mokokchung, Tuensung, Kohima and Wokha districts of Nagaland, Lohit and Dibang valleys, Chanlang and Papumpare districts of Arunachal Pradesh, Tamenglong district of Manipur and Coochbehar district of West Bengal (Singh *et al*. 2014) [1]. The silkworm produces golden hued silk which is famous for its luster, shining, toughness and durability and has high demand in golden market.
This silkworm is polyphagous in nature and feeds on a wide range of host plants (Choudhury 1970) [2]. Among the food plants, 'som' *Persea bombycina* Kost and 'soalu' *Litsea monopetala* Juss, are the two major primary food plants. Out of these two primary food plants 'soalu' *Litsea monopetala* Juss is semi-deciduous in nature while the other is evergreen. Muga silkworms are mostly wild unlike the mulberry silkworm, which is completely domesticated. Being wild in nature and outdoor nature of rearing these silkworms are exposed to various conditions of changing environment. Therefore, the muga silkworms succumb to various diseases which include pebrine, flacherie, muscurdine and grasserie. Outbreak of various diseases is the major constrains encountered in muga industry which ultimately affects silk production. Among these various diseases, flacherie is bacteria caused disease which is responsible for 40 % loss per annum to the silk industry. The term flacherie infers to the flaccid condition exhibited by the infected silkworm due to different ailments. Choudhury *et al*. (2002) [3] reported that flacherie disease in muga silkworm is caused by a bacteria *Pseudomonas aeruginosa* AC-3 strain. In our recent study 15 strains of bacteria were isolated from the gut of diseased muga silkworm of 3rd to 5thinstar and 5 strains were screened based on dominance. The strains responsible for flacherie disease were identified as *Pseudomonas. aeruginosa* (DRK1), *Ornithinibacillus bavariensis* (DRK2), *Achromobacter xylosoxidans* (KH3), *Staphylococus aureus* (FLG1) and *Bacillus thuringiensis* (MK1) as revealed by biochemical tests and 16S rRNA sequence analysis (Haloi *et al*. 2016) [5]. The pathogens primarily infect the alimentary tract of the silk worm, but high infection may affect the other system also. However, no further report on the effect of flacherie disease in silkworm gut architecture is available. Moreover, alaninaminotransferase (ALT) enzyme is conserved throughout evolution in almost all organisms. Activity level of this enzyme is considered as a specific clinical biomarker of cytotoxicity. Present study aims to investigate the effect of flacheie causing bacteria in ALT enzyme activity and alteration in histoarchitecture of gut structure of muga silkworm.

2. Materials and methods
2.1 Muga silkworm collection
Disease free layings (DFL) of muga silkworm were collected from Mangaldoi Seri culture firm, Assam. After hatching the 1st instar larvae were reared on *P. bombycina* (som plant) garden of IASST. Study was conducted during June – August 2015.

2.2 Microbial strains and culture

Microbial strains *Staphylococcus aureus* strain FLG1 (KR025521), *Bacillus thuringiensis* strain MK1 (KR069143) and *Pseudomonas aeruginosa* strain DRK1 (KP688076) isolated from the gut of flacherie diseased muga silkworm were used in this study (Haloi *et al*., 2016) [5]. The strains were allowed to grow at the nutrient agar plates and incubated overnight at 37 ^{0}C. LB broth medium was used to prepare the cell suspension of the bacterial strains by incubated at 37 ^{0}C for 48 h. Different concentrations of bacterial solutions were prepared by the method of Moar *et al*. (1995) [6]. The numbers of silkworms died at different concentration and time intervals (24-96 h) for the strains were recorded earlier. The LC_{50} values of *B.thuringiensis*, *P. aeruginosa* and *S. aureus* were calculated at different time intervals (24, 48, 72 and 96 h) within the range from 1.38 x10^2 to 3.63 x 10^7 CFU/ml. Based on the LC_{50} values for oral and injection experiments up to 72 h exposure were selected a sub-lethal dose of 10^3 CFU/ml (Haloi *et al*., 2016) [5]. A non-pathogenic *E. coli* strain (ATCC 25922) was used as positive control which was reported by Sauer and Moraru (2009) [7].

2.3 Alanineaminotransferase (ALT) assay

2.3.1 Crude enzyme preparation

For ALT assay the muga silkworm larvae were acclimatized in laboratory condition upto 4th instar larval stage and then the bacterial suspensions of all selected strains (10^3 CFU/larva) have been injected separately to the 5th instar larvae. A separate experiment for oral administration of each sub lethal concentration of bacteria suspension was also maintained. The whole gut of control, positive control and treated larvae was isolated after 24, 48 and 72 h time interval. The dissected gut was rinsed three times in 1X phosphate buffer saline (pH 7.4) and dissected into foregut, midgut and hindgut. Thereafter, the different gut sections of both control and treated were homogenized in 500 µl of 0.1 M sodium acetate buffer (pH 5.6) on ice and centrifuged at 15000 rpm for 15 min at 4 ^{0}C (Blakemore *et al.* 1995) [8].

2.3.2 Enzyme assay

ALT assay was carried out by adopting the standard method of Inagaki *et al.* (2012) [9]. Herein, 10 µl of the supernatant was mixed with 550 µl of the reaction mixture containing 0.5M L-alanine, 0.2mM NADH, 1.3U/ml lactate dehydrogenase and 0.9 mg/ml of BSA and vortexed. Thereafter, to the reaction mixture 50 µl of 180 mM 2-oxoglutarate solution was added and incubated for 90 min at 30 ^{0}C. Absorbance reading was measured at 339 nm, and the activity was expressed in unit (1U defined as change in absorbance of 0.001 per minute per µl of enzyme sample).

2.4 Histopathology study

The histopathological alterations in the gut of control, positive control and each bacteria infected 5th instar larvae were studied. The gut was dissected out and fixed in the fixative Carnoy's fluid (absolute alcohol: chloroform: glacial acetic acid = 6:3:1). Standard protocol of dehydration and paraffin embedding was applied for the preparation of histological slides (Gurr 1959) [10]. Five µm sections of gut tissues were cut in microtome machine and stained with hematoxylene and eosin solution. The prepared slides were observed under phase contrast microscope at 5 X, 10 X and 40 X resolutions (Zeiss Axio Cam ERC 5S).

3. Results

3.1 Alaninaminotransferase (ALT) assay

ALT enzyme activity was compared in control, positive control as well as bacteria infected groups. The control saline administered and positive control group did not show any significant alteration (Table 1). Oral administration experimental groups showed altered ALT activity than the control group. In foregut after 24 h of infection no significant alteration was observed. However, at 48 h except *S. aureus*, other two groups showed significant increase in ALT activity. Similarly, at 72 h of oral infection, all the experimental groups showed significant increase in ALT activity in comparison to control group (Fig. 1 a, Table 1). The midgut showed higher enzymatic activity than foregut and hindgut, however infected groups showed higher activity than control one. At 24 h, both *B. thuringienesis* and *P. aerogonisa* showed significantly higher ALT activity, however in *S. aureus* infected group the alteration was not significant. At 48 and 72 h, all the infected groups showed significantly higher level of ALT activity (Fig. 1 b, Table 1). In hind gut, all the infected groups showed significantly higher ALT activity at various time intervals of 24 – 72 h (Fig. 1 c, Table 1).

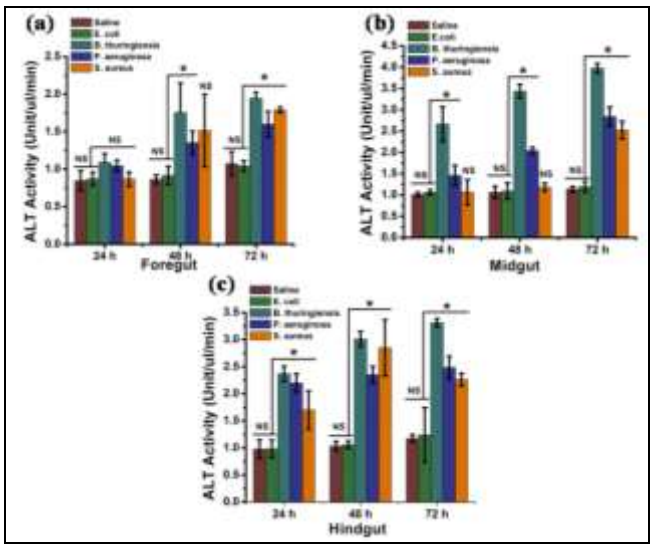

Fig 1: Comparison of alaninaminotransferase activity in saline administered control and orally administered bacterial infected groups of *A. assamensis* in different time intervals (a) foregut, (b) midgut and (c) hindgut (*P<0.01level of significance)

Table 1: Analysis of variance (ANOVA) of in saline administered control and bacteria administered groups in different parts of gut at various time intervals.

Oral administration	24 h		48 h		72 h	
	F	P	F	**P**	**F**	**P**
Foregut						
E. coli	0.0635	0.8133	0.4553	0.5368	0.0971	0.7708
B. thuringiensis	5.6393	0.0764	14.962	0.0180	72.4704	0.0010
P. aeruginosa	5.0187	0.0886	26.051	0.0069	15.1884	0.0175
S. aureus	0.0449	0.8424	5.3167	0.0824	58.4671	0.0015
Midgut						
E. coli	1.1138	0.3507	0.0455	0.8414	0.5029	0.5173
B. thuringiensis	48.777	0.0022	370.28	4.2983E-5	1471.308	2.757E-6
P. aeruginosa	10.008	0.0340	108.92	4.7615E-4	160.6673	2.230E-4
S. aureus	0.0955	0.7726	1.4416	0.29611	122.7210	3.776E-4
Hindgut						
E. coli	0.00244	0.96297	0.15191	0.71658	1.5408	0.2823
B. thuringiensis	119.021	4.0082E-4	421.359	3.32664E-5	1281.1632	3.636E-6
P. aeruginosa	76.8334	9.3385E-4	164.573	2.12835E-4	108.3128	4.814E-4
S. aureus	10.2300	0.03295	35.7404	0.00393	191.5652	1.572E-4

In bacteria injected group also higher ALT activity was observed than the control group, however the positive control group did not show any significant alteration. In foregut, 24 h of infection did not show significant alteration, however at 48 and 72 h significantly higher ALT activity was observed (Fig. 2 a, Table 2). The midgut and hindgut showed significantly higher ALT activity at 24 – 72 h of infection period (Fig. 2 b and c, Table 2).

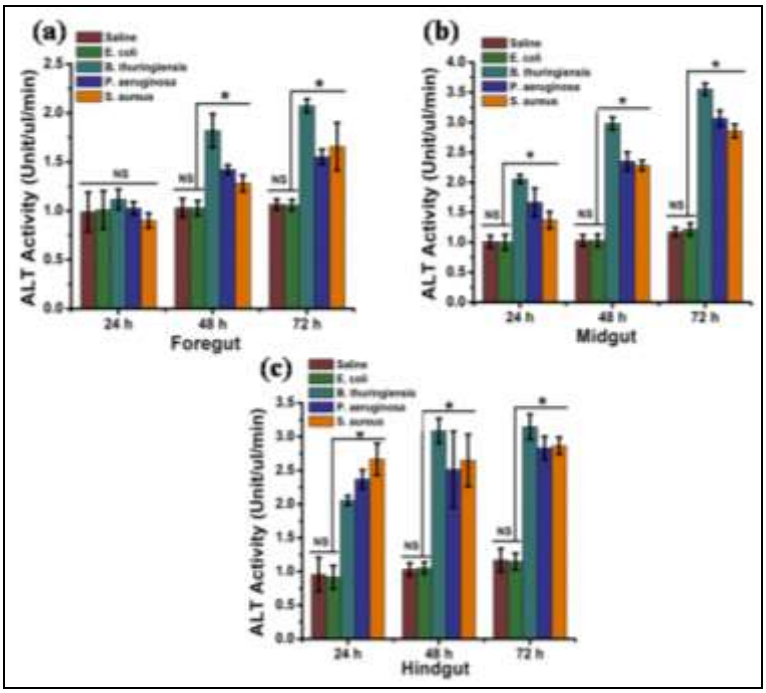

Fig 2: Comparison of alaninaminotransferase activity in saline injected control and bacteria injected groups of *A. assamensis* in different time intervals (a) foregut, (b) midgut and (c) hindgut (*P<0.01level of significance)

Table 2: Analysis of variance (ANOVA) of in saline injected control and bacteria injected groups in different parts of gut at various time intervals.

	24 h		48 h		72 h	
Injection	**F**	**P**	**F**	**P**	**F**	**P**
Foregut						
E. coli	0.015	0.906	9.211	0.992	0.022	0.887
B. thuringiensis	0.936	0.387	49.2	0.002	413.365	3.455E-5
P. aeruginosa	0.090	0.778	41.47	0.002	82.622	8.12E-4
S. aureus	0.518	0.511	11.96	0.02	16.921	0.014
Midgut						

E. coli	0.010	.924	6.881E-5	0.993	0.354	0.583
B. thuringiensis	219.431	1.20E-4	575.552	1.790E-5	1165.994	4.388E-6
P. aeruginosa	19.674	0.011	159.528	2.262E-4	477.776	2.592E-5
S. aureus	12.918	0.022	296.534	6.672E-5	491.296	2.452E-5
Hindgut						
E. coli	0.050	0.832	0.068	0.805	0.023	0.884
B. thuringiensis	53.874	0.001	295.553	6.716E-5	182.343	1.740E-4
P. aeruginosa	72.906	0.001	20.122	0.010	140.173	2.9137E-4
S. aureus	74.496	9.907E-4	49.467	0.002	190.418	1.5987E-4

1.1. Histopathological study

Both control and *E. coli* infected groups showed intact gut histoarchitecture. The gut lining was intact and no degenerative changes were observed in mucosa layer and microvilli structure. In oral administered groups alteration in gut line was observed. Moreover, degenerative changes in mucous layer and broken and fused microvilli were observed in each of the bacteria administered group (Fig. 3). However, in bacteria injected groups no such kind of changes was observed, therefore results only focused on oral administered group.

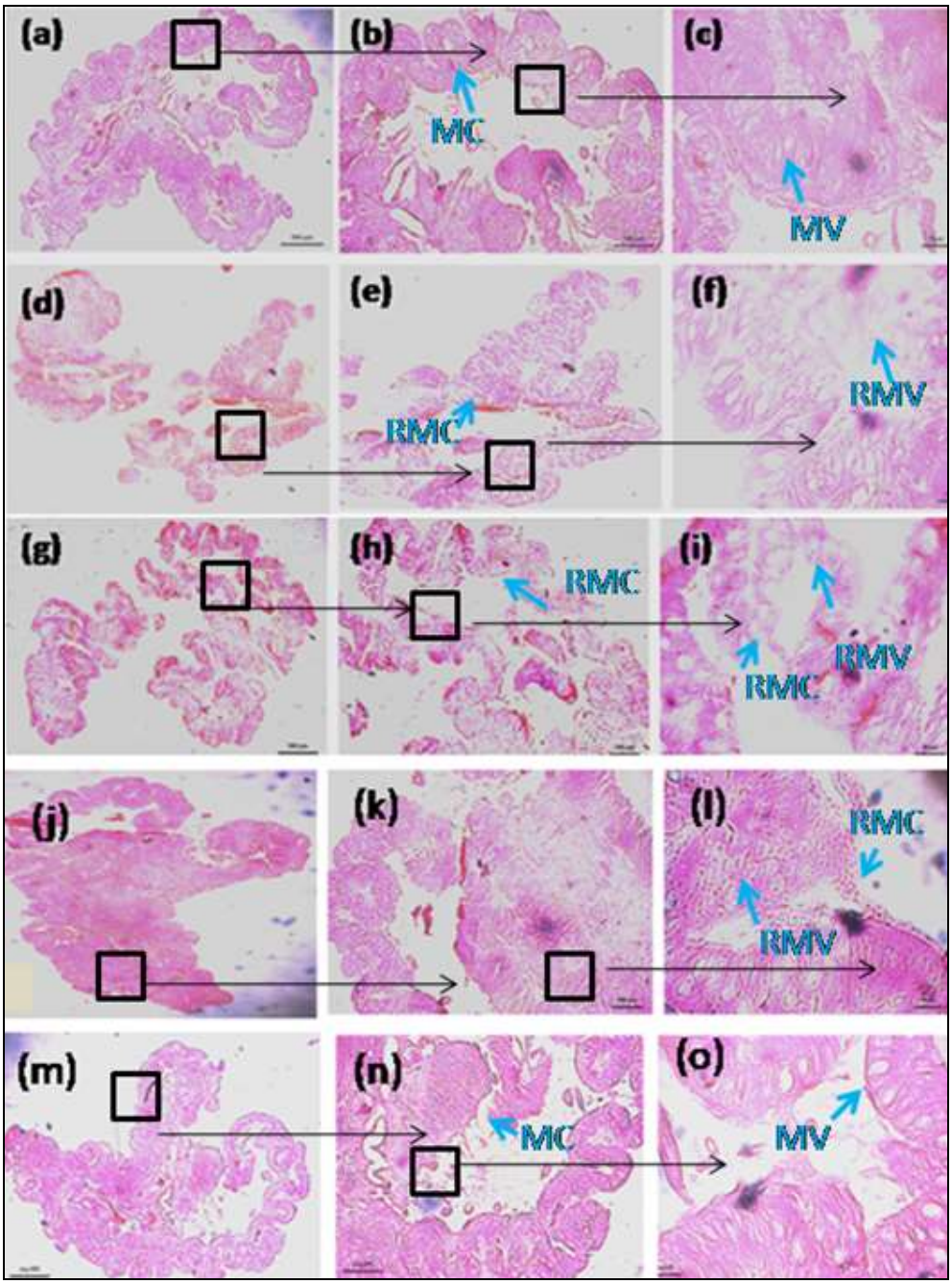

Fig 3: Histopathology of the midgut of 5th instar muga silkworm gut (a) 5 x, (b) 10 x and (c) 40 x saline administered control showing intact

gut lining, mucosa layer and microvilli MC- mucous layer, MV- microvilli (d) 5 x, (e) 10 x and (f) 40 x *B. thuringiensis* administered group(g) 5 x, (h) 10 x and (i) 40 x *P. aeruginosa* administered group (j) 5 x, (k) 10 x and (l) 40 x *S. aureus* administered group(m) 5 x, (n) 10 x and (o) 40 x *E. coli* administered group RMC- ruptured mucous layer, RMV- ruptured microvilli

4. Discussion

Sericulture practice is an agro-based industry which plays an important role in economy of the country. Assam is best known for the muga silkworm and golden hued muga fiber which is famous for its luster, shining, toughness and durability. Due to its wild nature only, outdoor rearing practice was performed as a result of which the muga larvae are susceptible to various diseases including flacherie disease. Flacherie diseased larvae showed symptoms like dysentery, retarded growth, sluggishness, flaccidity, loss of body luster, reddish black body color etc. which is quite visible from 3rd larval stage onwards. In our previous study isolation, characterization and pathogenicity assessment of gut associated bacteria of both healthy and flacherie infected muga silkworm wasperformed. *Pseudomonas aeruginosa* (DRK1), *Ornithinibacillus bavariensis* (DRK2), *Achromobacter xylosoxidans* (KH3) and *Staphylococcus aureus* (FLG1) strains were common in healthy as well as diseased larvae whereas, *Bacillus thuringiensis* (MK1) was found only in diseased larvae. Pathogenicity tests results also revealed that *S. aureus*, *P. aeruginosa* and *B.thuringiensis* which were isolated from diseased silkworm were highly pathogenic for the host among all the strains (Haloi *et al*. 2016) [5]. Moreover, inoculation test revealed that healthy larvaeshowed similar physiological changes like diseased silkwormswhen infected with these pathogenic strains (Choudhury *et al*., 2004; Mohanta *et al*., 2014) [4]. Since flacherie disease is mainly caused by bacterial contamination through food source, therefore toxicity in gut cannot be ignored. Activity level of alanineaminotransferase (ALT) enzyme is considered to be highly sensitive and fairly specific clinical biomarker of cytotoxicity (Inagaki *et al*. 2012) [9]. Present study results showed a significant increase in ALT activity in gut sections of both orally administered and injected groups. Increase in ALT activity is caused by leakage of this enzyme from injured tissue. Another hypothesis stated that an increase in lipid peroxidation leads to an increase in ALT activity. Significant increase in ALT activity often suggests the existence of a physiological challenge in body which includes microorganism infections, damage to some tissues by some toxic material etc. (Ender *et al*. 2005) [12].

In insects midgut is the important source of digestive enzymes as well as one of the main sites for the absorption of digested material (Vazquez-Arista *et al*. 1999) [13]. Tissue injury might lead to alteration of gut histology and present study deals with the gut structure of flacherie induced muga larvae. Herein, histopathology study indicated degenerative changes in gut lining which might be due to the presence of invader bacteria inside the gut.

5. Acknowledgement

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6. References

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