STREPTOCOCCUS MUTANS INFECTION AND ANTIBIOTIC-MEDIATED VARIATION IN THE ALANINE AMINOTRANSFERASE AND ASPARTATE AMINOTRANSFERASE ACTIVITY IN THE SILKWORM, *BOMBYX MORI*

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Abstract: *Streptococcus mutans* is one of the prime bacteria that cause dental caries in humans. The silkworm, *Bombyx mori* L. larvae infected with *S. mutans* exhibit characteristic disease symptoms indicating successfull induction of pathogenesis for the first time. However, its impact on biochemical and physiological processes in the host is unknown. Hence, we have planned to uncover the impact of *S. mutans* infection and antibiotics in the regulation of alanine and aspartate enzyme activity in the silkworm haemolymph. The results revealed that *S. mutans* infection through haemocoel and oral routes accelerated the activity of Aspartate Aminotransferase **(**AST) at the rate of 154.37 and 183.33% respectively. Similarly, the activity of Alanine Aminotransferase (ALT) was also found elevated at the rate of 143.15 and 181.73% in comparison with uninfected larvae. Administration of antibiotics – Advent and Taxim through oral route restored the larval health by declining the AAT enzyme activity accounting to 62.66 and 46.69% in the haemolymph. Through haemocoel mode of administration of antibiotics - Advent and Taxim, the highest reduction in AST activity accounting to 20.77 and 16.44% respectively were recorded. Concomitantly, ALT activity in the haemolymph was found to be declined at the rate of 53.07 and 41.63% in the larvae administered with Advent and Taxim antibiotics through the oral route, while 41.55 and 27.70% of reduced enzyme activity was noticed for haemocoel route of administration. Taken together, it was obvious that while *S. mutans* infection enhanced the AST and ALT activity, the antibiotics declined the activity of these enzymes. So, we propose silkworm as a potent model system and AST and ALT as marker enzymes to evaluate novel drugs against *S. mutans* and other pathogenic infection.

Index terms : Alanine aminotranferase, Aspertate aminotransferase, Silkworm, *S. mutans*

I. INTRODUCTION

In recent years, the silkworm, *Bombyx mori* L. has emerged as a promising model to investigate several human diseases and disease-causing agents such as bacteria, virus, fungi etc., and drug toxicity (Sekimizu, 2012). By and large, the silkworm larvae are susceptible to several diseases caused by the infection of a wide range of microbes. Of which, *Streptococcus faccalis* and *S. liquifacions* induce a disease commonly called - flacharie in the silkworm (Karthikairaj *et al.,* 2013), but *S. mutans*, which is known for causing dental caries in human, pathogenesis in the silkworm larvae is remaining unexplored. With this gap, *S. mutans* - *B. mori* disease model was developed (Likhith gowda *et al.,* 2019) but information on biochemical impact has not been available that warranted systematic investigation.

However, *S. mutans* not only causing dental caries in humans but also responsible for the onset of many systemic diseases, such as cardiovascular disease, bacterial pneumonia, diabetes mellitus, and low birth weight (Xiaojing *et al.,* 2000). These systemic conditions could be initiated or detrimentally influenced by the repeated entry of bacteria into the bloodstream (Kinane, *et al.,* 2005; Jan Beck *et al.,* 1996). Further, there are few reports stating that the liver is the target organ for the specific strain of *S. mutans* mediated aggravation of colitis (Kojima, *et al.,* 2012). Since there are several tissues and organs found in the silkworm are analogous to mammals (Kaito *et al.,* 2002), the fat body - an organ functionally resembles the mammalian liver and adipose tissue (Yasuhiko Matsumoto, 2015). So, the tissue injury caused due to hepatotoxic drugs can increase the activity of marker enzymes in the blood of human (Ozer *et al.,* 2008), which remain enigmatic in the silkworm. Similarly, the impact of *S. mutans* and toxic drugs might also induce tissue injury in silkworm, which can be correlated with the human system. So such

physiological consequences of *S. mutans* infection in the silkworm, since unclear, it needs to be investigated for the use as a model for *S. mutans* infection to understand the biochemical process involved in host-pathogen interaction. To measure the rate of tissue damage due to pathogens infection, AST and ALT enzyme activity shall be used as a marker enzymes and evaluate the efficacy of novel antibiotics against *Streptococcus* sps. and other pathogenic microorganisms.

II. MATERIALS AND METHODS

Experimental animal, Bacteria and Antibiotics

Healthy fifth instar larvae of silkworm (*Bombyx mori*) strain NB_4D_2 was used to induce the infection. To this, *Streptococcus mutans* a pure culture (MTCC 890) was procured from microbial type cell culture and gene bank, Chandigarh, India. Antibiotics - Advent, *i.v.* (Amoxycillin + potassium clavulanate injection IP) and Taxim, *i.v/i.m.* (Cefotaxime sodium injection IP), which are advocated for odontogenic infection was purchased from a registered local pharmacy.

Preparation of *S. mutans* inoculum

According to the supplier's protocol, the lyophilized *S. mutans* received from gene bank was rejuvenated using Brain-Heart-Infusion (BHI) broth. Serial dilution was performed from 2.1 x 10^{10} CFU/ml to 2.1 x 10^4 CFU/ml based on the optical density reading at 600 nm (Elico SA 165, India) using 0.5 McFarland (1.5 x 10^8 CFU/ml) as standard and incubated for 12 hr. Further, the viable cells from each concentration were isolated by adding the required quantum of 0.3% normal saline and centrifuged at 4000 rpm for 10 min at 4^0C. To derive the required density of bacterial suspension, cells isolated were re-suspended in 1 x Phosphate Buffer Solution (PBS) for each of serial concentration.

In vivo test for the action of antibiotics against *S. mutans* - haemocoel route

Healthy fifth instar larvae weighing equally were selected and divided into seven treatment groups with three triplications and 10 larvae in each replication. T2-T6 groups of larvae were injected with bacterial suspension through haemocoel, while T1 injected with PBS and T0 maintained as absolute control without any injection. 12 h was maintained as lag period and known quantity of Advent and Taxim antibiotics, which were dissolved in sterile water provided by the manufacturer to make different concentrations was administered to different treatment groups of the larvae.

In-vivo test for the action of antibiotics against *S. mutans* - Oral route.

For the oral route of administration of *S. mutans* and antibiotics, newly exuviated (after 4^{th} moult) silkworm larvae having similar weights were selected and divided into 15 treatment groups in triplicates with 10 larvae in each replication. The groups T2 – T14 were provided mulberry leaves smeared with bacterial culture, whereas, control groups (T0 and T1) were fed with fresh mulberry leaves. Antibiotics prepared by dissolving 2.50 and 5.0 µg of Advent® and Taxim® in sterile water (as per the manufacturer instructions) were smeared on the mulberry leaves and fed to the larvae after drying for 10 min in room temperature. A control group of larvae were fed with untreated leaves. Similarly, the second and third dose of antibiotics were administered to the experimental larvae at 48 and 72 hours post inoculation (hpi) respectively. All the experimental larvae were maintained in the same environmental conditions till cocooning.

Haemolymph collection

The first proleg of treated and control group larvae was incised and haemolymph was collected separately in precooled vials containing a few crystals of thiourea (to prevent oxidation of haemolymph). The haemolymph sample was centrifuged at 3000 rpm for 10 min at 4° C, and the supernatant was used to estimate AST and ALT enzyme activity (Takai and Tamashiro, 1975).

Estimation of aspartate amino transferase activity (AST)

The activity of AST in the haemolymph was estimated following the method of Reitman and Frankel (1957) using pyruvic acid as standard. After incubation at 37^oC for 1 h, the enzyme substrate mixture was treated with 2, 4-dinitrophenyl

hydrazine solution and then 0.4 N sodium hydroxide was added. After 10 min, intensity of the colour developed was measured at 520 nm using spectrophotometer that represents the amount of oxaloacetate formed as an index of AST level. It was computed based on the standard curve and accordingly, the results were expressed in µg of oxaloacetate per ml of haemolymph (Lakshmi and Benarjee, 2016).

Estimation of alanine aminotransferase activity (ALT)

The ALT activity was estimated in the haemolymph following the method of Reitman and Frankel (1957) using pyruvic acid as standard. The enzyme substrate mixture after incubation (37°C for 1 h) was treated with 2, 4-dinitrophenyl hydrazine solution and then 0.4 N sodium hydroxide was added. After 10 min, intensity of the colour developed was measured at 520 nm in spectrophotometer that represents the amount of pyruvate formed as an index of ALT level. It was computed based on the standard curve and accordingly, the results were expressed in µg of pyruvate per ml of haemolymph.

Statistical analysis

The data obtained in the current investigation was subjected to one-way ANOVA analysis ($p \leq 0.05$) employing SPSS statistical package (ver. 21.0).

III. RESULTS

***S. mutans* and antibiotics induced changes in the AST enzyme activity - haemocoel route**

The AST activity 2.63 µg/ml recorded in the haemolymph of healthy larvae was found increased to the level of 6.69 µg/ml after *S. mutans* infection. Interestingly, noticable rate of decline in the AST enzyme activity was observed after antibiotic - Advent injection to the haemocoel that differs with the concentrations administered into the larvae. Advent at the concentration of 2.50 and 5.0 µg/larva treated batches exhibit 5.85 and 5.30 µg/ml of enzyme activity respectively (Fig. 01). In contrast, the AST enzyme activity was found high in Taxim compared to Advent treated batches. Declined AST enzyme activity measuring 5.59 µg/ml was recorded in the larval batches treated with 2.50 and 5.0 µg/larva of Taxim against 6.69 µg/ml of enzyme activity in *S. mutans* infected larval batches (Fig. 1). All these data are statistically significant at $p<0.05$ (Table 1).

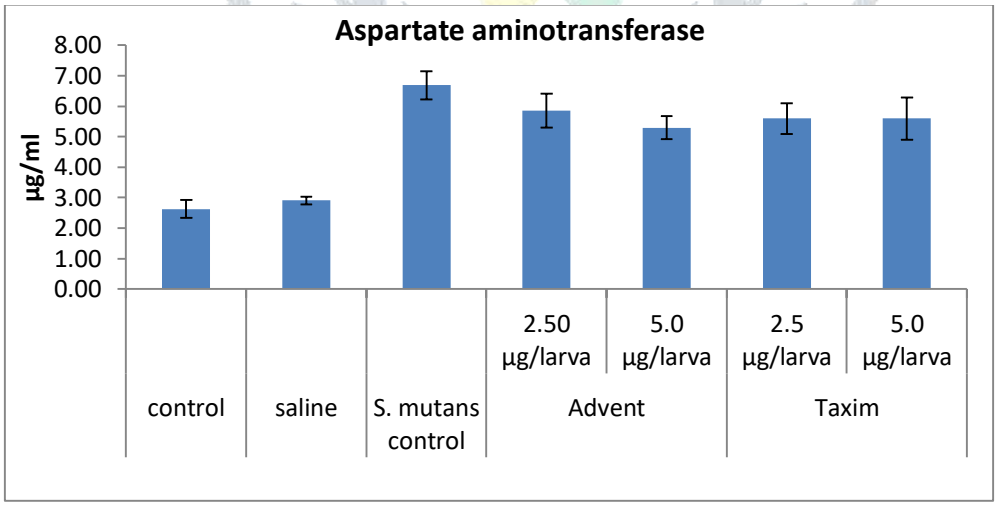

Figure 1. Changes in the silkworm haemolymph aspartate aminotransferase activity due to antibiotics against *Streptococcus mutans* infection administered through haemocoel route.

Changes in the activity of AST in the haemolymph of larvae infected with *S. mutans* and influenced by antibiotics - oral route

The AST activity recorded from *S. mutans* infected larval batches was 8.33 µg/ml, which is lesser than the control batches (2.94 µg/ml). The AST activity was declined after antibiotic treatment and the lowest AST activity of 3.11 µg/ml was recorded in the batch treated with three doses of 5.0 µg/larva/dose of Advent, while it was 6.15 and 4.44 µg/ml for a single and two doses of Advent. Interestingly, in the lower concentration (2.50 µg/larva/dose), the AST activity was declined to 7.50, 6.47 and 5.13 µg/ml in the larval batches received a single, two and three doses of 2.50 µg/larva/dose of Advent respectively. All these data are statistically significant at $p<0.05$ (Table 1).

Notably, the AST activity was increased to 8.81 µg/ml in the larval batches, which received a single dose of Taxim at the concentration of 2.50 µg/larva/dose. Further, the AST activity was reduced to 7.81 and 7.69 µg/ml in two and three doses of antibiotic-treated batches at the same concentration respectively. Consequently, as the concentration of antibiotic increases to 5.0 µg/larva/dose, the enzyme activity was further declined to 5.94, 5.17 and 4.44 µg/ml in the larval batches received a single, two and three doses of Taxim respectively (Fig. 03; Table 01). All these data are statistically significant at $p<0.05$ (Table 1).

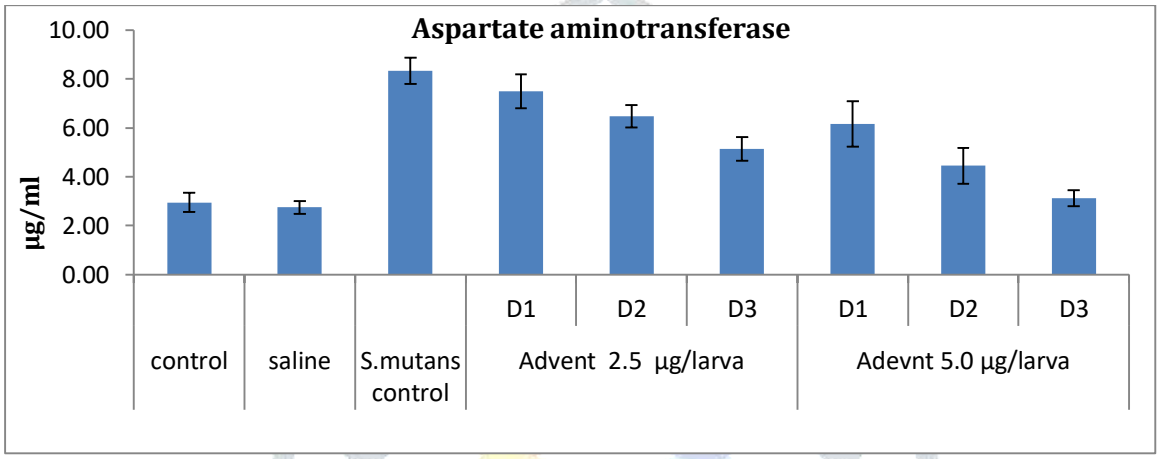

Figure 2. Changes in the haemolymph aspartate aminotransferase activity due to antibiotic - Advent against *Streptococcus mutans* infection administered through oral route.

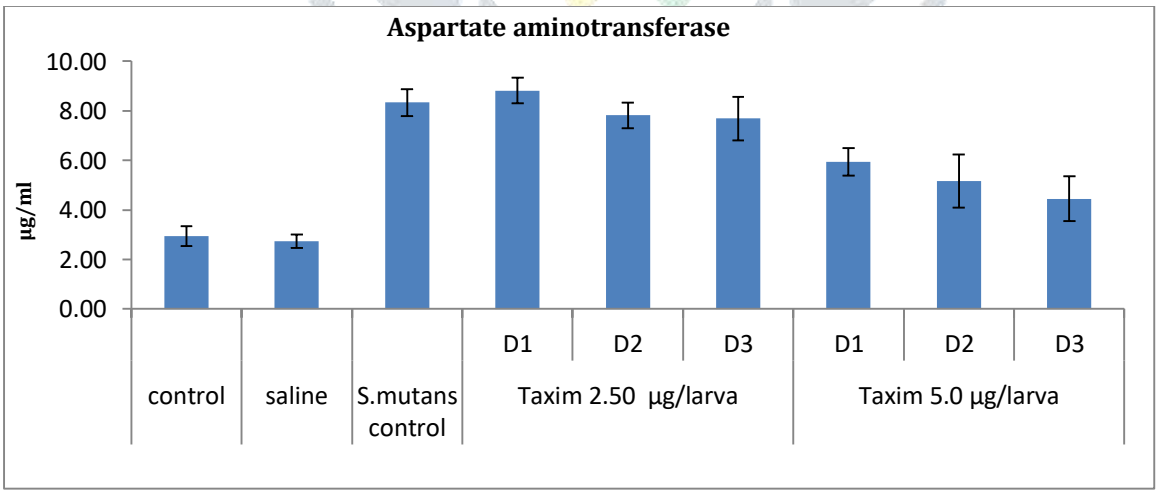

Figure 3. Changes in the silkworm haemolymph aspartate aminotransferase activity due to antibiotic - Taxim against *Streptococcus mutans* infection administered through oral route.

Regulation of ALT activity in the hemolymph due to the antibiotic administration in *S. mutans* infected silkworm larvae through intra haemocoel route.

The ALT activity was found increased being highest as 4.62 µg/ml in the *S. mutans* infected larvae against 1.90 µg/ml in the uninfected larvae. After antibiotic - Advent treatment, the enzyme activity was gradually declined to 2.70 and 3.42 µg/ml in the larval batches received 2.50 and 5.0 µg/larva of Advent (Fig. 4). Taxim treated batches also exhibit similar trend with lowered ALT activity of 3.34 and 3.45 µg/ml in the haemolymph of silkworm larval batches received 2.50 and 5.0 µg/larva of Taxim respectively.

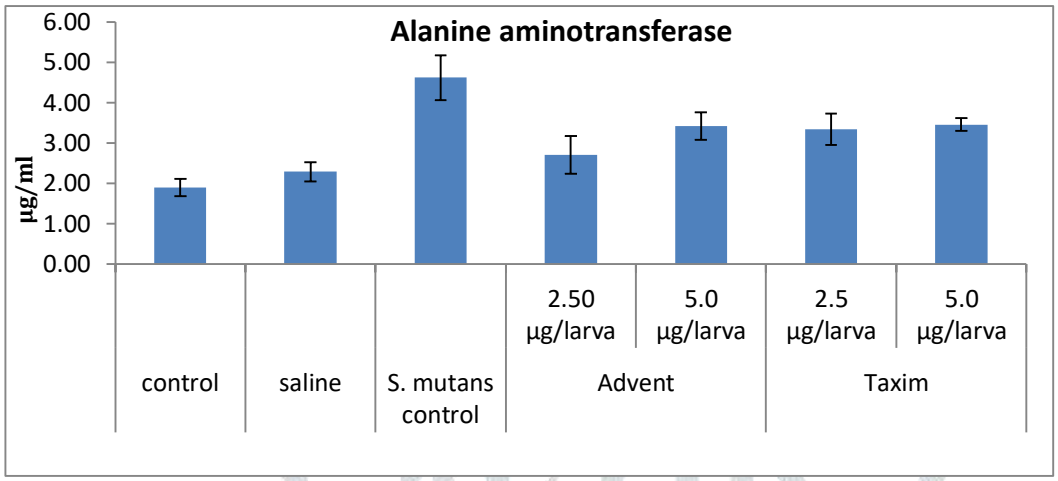

Figure 4. Changes in the silkworm haemolymph Alanine aminotransferase activity due to antibiotics against *Streptococcus mutans* infection administered through haemocoel route

Changes in the activity of ALT in the haemolymph of the silkworm larvae infected with *S. mutans* and the impact of antibiotics administered through oral route

The activity of yet another key enzyme ALT was also the same as that of AST. The lowest ALT enzyme activity was recorded in the haemolymph of uninfected larvae was 2.08 µg/ml, but it drastically increased to 5.86 µg/ml after *S. mutans* infection. Ensuing antibiotic treatment reduced the ALT enzyme activity to 5.64, 4.63 and 3.78 µg/ml in the larval batches treated with a single, two and three doses of 2.50 µg/larva/dose of Advent. A reduction trend was further continued in the higher concentration treated batches with the lowest enzyme activity of 2.75 µg/ml in the larval batches received three doses of 5.0 µg/larva/dose. For a single and two doses of antibiotic, the ALT enzyme activity was 4.75 and 3.61 µg/ml respectively (Fig. 5).

A considerable reduction in the ALT enzyme activity was recorded after treating *S. mutans* infected larvae with Taxim antibiotic, although, it was not greater than Advent. The lowest ALT activity of 3.42 µg/ml was recorded in the haemolymph of larval batches treated with three doses of 5.0 µg/larva/dose of Taxim antibiotic. With the administration of a single and two doses of antibiotic, the activity was 4.47 and 4.00 µg/ml respectively. However, for 2.50 µg/larva/dose of antibiotic treatment, the lowest ALT enzyme activity of 5.67 µg/ml was recorded from the three doses followed by two and a single dose that account to 6.00 and 6.25 µg/ml respectively. These results are statistically significant at $p<0.05$ (Fig. 06; Table 01).

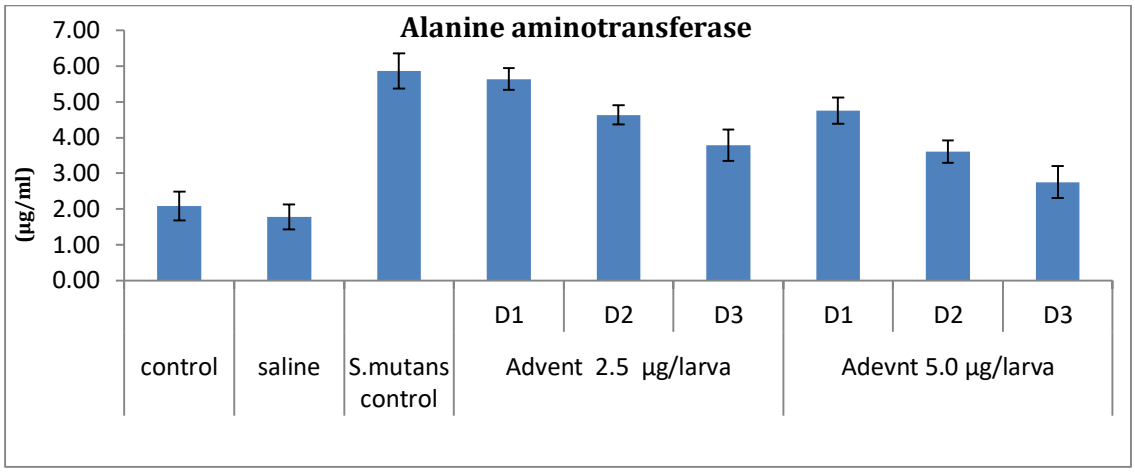

Figure 5. Changes in the silkworm haemolymph alanine aminotransferase activity due to antibiotic - Advent against *Streptococcus mutans* infection administered through oral route

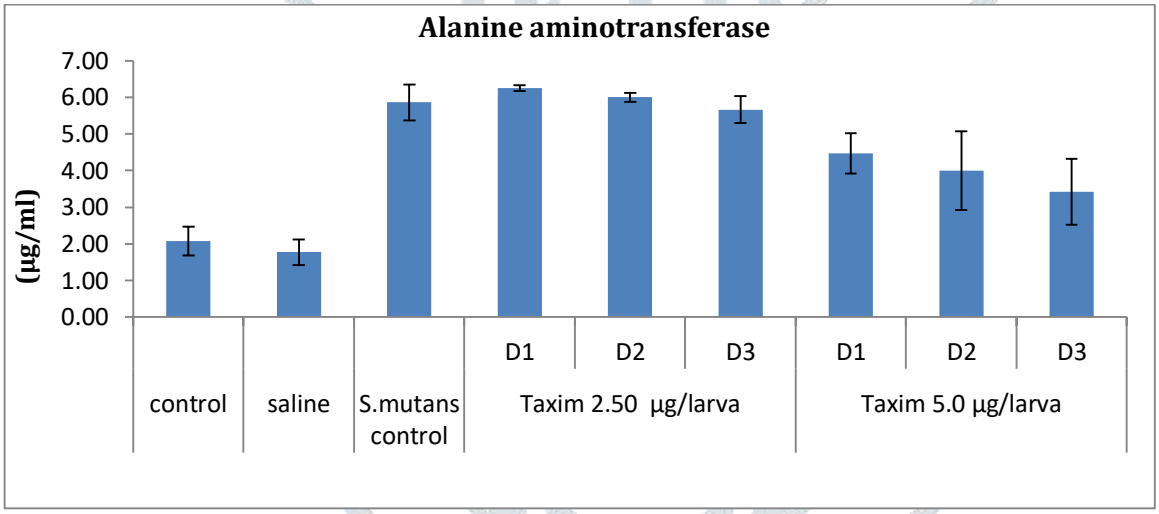

Figure 6. Changes in the silkworm haemolymph alanine aminotransferase activity due to antibiotic - Taxim against *Streptococcus mutans* infection administered through oral route

Table 1. Statistical analysis of the data for the changes in the AST and ALT enzyme activity as affected by *Streptococcus mutans* infection and antibiotics administered through haemocoel and peroral route.

Route of administrations	Antibiotics	AST		ALT	
		F-value	Significance ($p \leq 0.05$)	F-value	Significance ($p \leq 0.05$)
Haemocoel	Advent	67.20	0.000**	36.85	0.000**
	Taxim	67.20	0.000**	36.85	0.000**
Oral	Advent	76.549	0.000**	89.15	0.000**
	Taxim	70.04	0.000**	153.62	0.000**

IV. DISCUSSION

The transaminases are the important components of amino acid catabolism, which mainly involved in transferring an amino group from one amino acid to another Keto acid. The AST and ALT serve as a strategic link between carbohydrate and protein metabolism. Commonly, ALT activity is mentioned as an index for the breakdown of amino acids and AST as a sign for entrance of amino acid to glucogenesis process. Glucogenesis is the main path for sugar synthesis from non-carbohydrate substrates (Lehninger, 1982) and are known to be altered during various physiological and pathological conditions (Etebari *et al*., 2005). Upon *S. mutans* infection, the ALT and AST activity were greatly altered in the haemolymph of silkworm larvae. *S. mutans* infection through both haemocoel and oral routes increases the activity of ALT enzyme at the rate of 143.41 and 181.30%, whereas, 154.23 and 183% in AST enzyme against uninfected batches. The increase in activity of aminotransferases, in turn, increases the supply of precursors to Krebs cycle which finally results in the production of energy (Anitha, 2010). The results obtained in the present study is congruent with the previous reports wherein an abnormal increase in the concentration of glutamic aspartate transaminase and of glutamicalanine transaminase activities was reported in the nuclear polyhedral virus infected silkworm (Ramaiah and Veerabasappa 1970).

Increased alanine aminotransferase (ALT) activity in mammalian blood is due to leakage of this enzyme from injured tissue. Therefore, increased ALT and AST enzyme activity in the *S. mutans* infected larvae might be because of the damage caused to fat body cells. This hypothesis strongly supports the pathogenicity of *S. mutans* in the fat bodies of the silkworm, which is similar to that of the human liver. Therefore, a strong correlation was established between the human and silkworm with respect to *S. mutans* infection, substantiating the silkworm larvae as a promising model for human dental caries disease model developed by Likhit gowda and Manjunatha (2019). Interestingly, after antibiotic treatment, a substantial decrease in the ALT and AST activity was recorded, which clearly acts as marker enzyme to demonstrate the recovery of cellular machinery from the *S. mutans* infection and perform normal biochemical process. These findings indicate the recovery of silkworm larvae from the *S. mutans* infection and acquire normal functional abilities. However, during the course of evolution, ALT is conserved in invertebrates and vertebrates (Ozer *et al.*, 2008) and is therefore considered to be a surrogate marker of tissue injury in insect larvae. Hence, we postulate that the silkworm shall be a valuable model not only to evaluate the pathogenicity of bacteria but also the toxic effects of chemical compounds that are pharmaceutical important considering ALT and AST as marker enzymes, which unique feature of the study.

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