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# BIOMOLECULAR CHANGES DUE TO *STREPTOCOCCUS MUTANS* INFECTION AND ANTIBIOTICS TREATMENT IN THE HUMAN DENTAL CARIES - SILKWORM (*BOMBYX MORI*) DISEASE MODEL

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**Keywords:**

Antibiotics, Haemolymph, Protein, Silkworm, *Streptococcus mutans*, Trehalose

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**ABSTRACT:** Changes in the hemolymph protein and trehalose contents of the silkworm, *Bombyx mori* L. due to *Streptococcus mutans* infection and antibiotics treatment was evaluated. Interestingly, *S. mutans*, which was successfully grown in the body of the larvae led to decline 69.60 and 50.21% of protein content in the hemolymph. Concomitantly, there was a drastic rise in the content of trehalose to 60.22 and 65.13% due to bacterial infection-induced through hemocoel and oral routes respectively compared to healthy larval batches. Antibiotics clinically advocated to cure dental caries was substantially lowered the bacterial infection in the silkworm larvae regulating a rise in the protein and reduction in the trehalose contents. Notably, antibiotic-Advent treatment through hemocoel and oral routes explicit higher rate of recovery in the synthesis of protein that amounts to 133.79 and 92.62%, while trehalose content was found 34.30 and 40.31% less in the hemolymph that equivalent to healthy larvae respectively. Similarly, taxim was also influenced for an elevation of protein content 106.73 and 78.99%, whereas there was a reduction of 24.47 and 33.70% in the trehalose content of the hemolymph in the larvae received antibiotics through hemocoel and oral routes respectively. Among the two antibiotics, the efficacy of advent was much superior over taxim, despite both the antibiotics showed dose, concentration and drug dependent effects against *S. mutans* infection by envisaging that silkworm larvae shall be the promising human dental caries disease model to evaluate novel drugs for its toxicity and efficacy before subjecting them for clinical trials.

**INTRODUCTION:** *Streptococcus mutans* is gram-positive cocci shaped bacterium commonly found in human dental caries and such animal models [1], which is mesophilic and grow at a temperature ranging from 18-40 degree Celsius.

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It ferments sugar for energy and produces an acidic environment that demineralizes the superficial structure of tooth by which damage the dental hard structures in the vicinity of fermentable carbohydrates [2]. The empirical evidence revealed that this bacterium not only causes oral infection but also responsible for several systemic diseases.

Primarily, it inhabits in the oral cavity, but capable of invading pharynx and intestine due to the poor and specific immune system of the oral mucosa. The bacteria can also attack the circulatory system with ease and cause endocarditis and massive

bacteraemia, leading to the systemic diseases [3]. Since there are different bacterial communities involved in the onset of dental caries, it is essential to understand the disease biology to develop appropriate curative strategies [4]. The silkworm, *Bombyx mori* L. is an extremely vital model system, which is providing a significant insight to explore many areas of biomedical sciences. Right from the nineteenth century, *B. mori* has been serving as a model organism for varied aspects of biological sciences and has contributed many classic paradigms for understanding genetics and molecular biology [5].

Consequently, it has become one of the exponentially studied Lepidopteran insects in the field of physiology, biochemistry, pathology, and genetics [6] and considered as a central model organism of Lepidopterans [7]. Besides, in recent years, silkworms have been established as an infection model for various human virulent microorganisms like - *Staphylococcus aureus*, *Pseudomonas aeruginosa*, *Vibrio cholerae*, *Stenotrophomonas maltophilia*, *etc.*, [8, 9] except *S. mutans* that open ample scope for the development of silkworm as human dental caries disease model[10].

By and large, protein synthesis in silkworm is depending on the nutritional status during larval development [11]. Proteins are important for the development, metamorphosis and maintain a number of physiological functions [12]. On the other hand, trehalose is a major sugar in the larval hemolymph, which is synthesized in the fat bodies by utilizing sucrose, glucose, fructose, maltose, cellobiose, and sorbitol-derived from diet [13]. These biomolecules are responsible for supplying nutrients to maintain normal growth and development of silkworm larvae and subsequent stages of its life cycle. Hemolymph cells perform host defense through phagocytosis and signaling and employ a form of self-non-self recognition that is independent of microbial patterns [14].

Thus, the progressive multiplication of *S. mutans* in the host system and effectiveness of antibiotic against the pathogen, which is associated with specific metabolic process coupled with corresponding biochemical changes in the infected tissues of the silkworm, *B. mori* larvae were investigated and presented. The chief aim of this study was to establish a strong biochemical evidence to envisage the silkworm larvae, as a promising human dental caries (*S. mutans*) disease model.

## MATERIALS AND METHODS:

**Experimental Animal, Bacteria, and Antibiotics:** The healthy fifth instar silkworm, *Bombyx mori* (strain $NB_4D_2$) larvae were used for the induction of *Streptococcus mutans* infection. The pure cell culture of bacteria (MTCC 890) was procured from microbial type cell culture and gene bank, Chandigarh, India. Commercially available antibiotics advocated in odontogenic infections, Inj. Advent, *i.v.* (Amoxicillin + potassium clavulanate) Inj. and Taxim, *i.v/i.m.* (Cefatoxin) was purchased from a registered local pharmacy.

**Rearing and Maintenance of Silkworm Larvae:** The silkworm eggs were incubated under optimum environment condition (25 ± 1 ºC temperature and 75 ± 5% relative humidity) followed by black-boxing on the 8th day until hatching in the laboratory. The larvae were reared on mulberry leaves following standard rearing procedure [15].

**Maintenance and Harvesting of *S. mutans*:** The lyophilized *S. mutans* were rejuvenated according to supplier's protocol in an aseptic environment in Brain-Heart-Infusion (BHI) broth and its growth was assessed based on absorbance at 550 nm using UV-Vis spectroscopy.

Further, 20 µL of the sample was cultured on the surface of BHI agar and incubated for 48 h for colony development that gave rise to 210 colonies. This was re-suspended in BHI broth to acquire $2.1 \times 10^{10}$ CFU/mL and considered as bacterial stock suspension. Through dilution, $2.1 \times 10^4$ CFU/mL concentration was prepared based on the optical density at 600 nm (Elico SA 165, India) using 0.5 McFarland ($1.5 \times 10^8$ CFU/mL) as standard and incubated for 12 h. Further, the viable cells from each concentration were isolated by adding the required quantum of saline and centrifuged at 4000 rpm for 10 min at 4 ºC. Bacterial cells isolated were re-suspended in 1 × phosphate buffer solution (PBS) to derive the required density of bacterial suspension for further experiments.

**Intra-Haemocoel Route of Administration of *S. mutans* and Antibiotics:** Healthy fifth instar silkworm larvae having equal weight on day-3 were divided into seven treatment groups in triplicate with 10 larvae in each replication. A control group of healthy larvae was maintained without any inoculation. The other group of larvae was injected with saline. A group of larvae was injected with 30 µl of *S. mutans* suspension that could induce $LC_{50}$ (a detailed procedure is protected due to patenting). After a lag period of 12 h, appropriate quantity of selected antibiotics (for each concentration) was dissolved in sterile water provided by the manufacturer. Further, different concentrations of antibiotics were injected into different groups of larvae.

**Oral Route of Administration of *S. mutans* and Antibiotics:** To test dose-dependent effectiveness of commercially available antibiotics against *S. mutans*, three doses of Advent and Taxim were administered to the silkworm larvae along with mulberry leaves. To this, fifth instar silkworm larvae having similar weights were divided into 15 treatment groups in triplicates with 10 larvae each (the detailed procedure is protected in lieu of a patent). They were provided with mulberry leaves smeared with bacterial culture, whereas the control groups were fed with fresh mulberry leaves.

After 12 h of post-infection (hpi), a precise quantum of antibiotics (for each concentration) was dissolved in a defined volume of sterile water provided by the manufacturer and then smeared on mulberry leaves and allowed to dry for 10 min at room temperature. The antibiotic-treated leaves for different test batches and untreated leaves in the control groups were fed. On day-3 and day-4 of the fifth instar, 2nd and 3rd dose of antibiotic-treated leaves were fed to different test batches and untreated leaves for the control group. All these larvae were reared in the same environmental conditions until spinning following standard rearing procedure [15].

**Collection Haemolymph:** Haemolymph was collected in pre-cooled vials containing a few crystals of thiourea (to prevent oxidation of hemolymph) by cutting the first proleg of treated and control group larvae separately. The hemolymph was centrifuged at 3000 rpm for 10 min at 4 ºC, and the supernatant was used for the biochemical analysis. With the same hemolymph sample, both protein and trehalose contents were estimated.

**Estimation of Protein:** Silkworm hemolymph protein was estimated by Lowry's method [16] using crystalline bovine serum albumin (BSA) as standard. To 0.1 ml of the hemolymph sample, 0.9 ml of distilled water was added, followed by the addition of 5 ml of protein reagent. The tubes were kept for 15 min at room temperature. Then 0.5 ml of Folin's reagent was added, and the tubes were allowed to stand for 30 min. The spectrophotometer absorbance at 660 nm was recorded and the concentration of protein in the sample was calculated with the standard curve. The results were expressed in mg of protein per ml of hemolymph.

**Estimation of Trehalose:** Estimation of hemolymph trehalose was carried out according to the method of Roe (1955) [17]. A known quantity of hemolymph was collected in each test tube and 0.5 ml of 20% NaOH was added. After shaking, the tubes were kept in boiling water for 10 min then the tubes were cooled in room temperature. Then 5 ml of anthrone reagent (0.05% anthrone in 70% sulphuric acid) was added to the tubes, and once again kept in boiling water for 15 min for the development of color. Then the tubes were cooled at room temperature. The intensity of color was measured on a spectrophotometer at 620 nm keeping trehalose as a reference standard.

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

## RESULTS:

### Quantitative Changes in the Total Protein Content of Haemolymph in the *S. mutans* and Antibiotics Treated Larvae of the Silkworm, *B. mori*:

**Administration of *S. mutans* and Antibiotics - Haemocoel Route:** *S. mutans* infection-induced through hemocoel altered the total protein content in the hemolymph of the silkworm larvae that drastically was found depressed to 23.17 mg/ml as against 76.20 mg/ml in control batches. Interestingly, after administration of 2.50 and 5.0

µg/larva of Advent in the same route, the protein content was increased to 54.17 and 50.13 mg/ml respectively. Whereas in case of Taxim, 47.90 and 47.57 mg/ml protein content recorded was from the larval batches treated with the concentrations of 2.50 and 5.0 µg/larva respectively, which is considerably less to Advent but remarkably higher than that of *S. mutans* infected larvae **Fig. 1**. Statistically, the changes recorded with respect to the total amount of hemolymph protein derived from all the batches - control, *S. mutans* infected and antibiotic-treated batches were significant at $p<0.05$ **Table 1**.

**Administration of *S. mutans* and Antibiotics - Oral Route:** The manifestation of total protein content in the hemolymph was noticed due to *S. mutans* infection through oral route of administration to the silkworm larvae. The total amount of hemolymph protein recorded in the healthy (control) larvae was 88.83 mg/ml, but it was declined to 44.23 mg/ml after *S. mutans* infection. Eventually, after antibiotics treatment, there was a significant recovery in the protein synthesis with the highest record of 85.20 mg/ml in the larval batches treated with three doses of 5.0 µg/larva/dose of Advent. For a single and two doses of antibiotic, the protein content recorded was 57.50 and 75.97 mg/ml respectively. But for lower concentration (2.50 µg/larva/dose), 53.87, 68.37 and 82.43 mg/ml of protein content was recorded in the larval batches treated with a single, two and three doses of Advent antibiotic **Fig. 2**. All these data are statistically significant at $p<0.05$ **Table 1**.

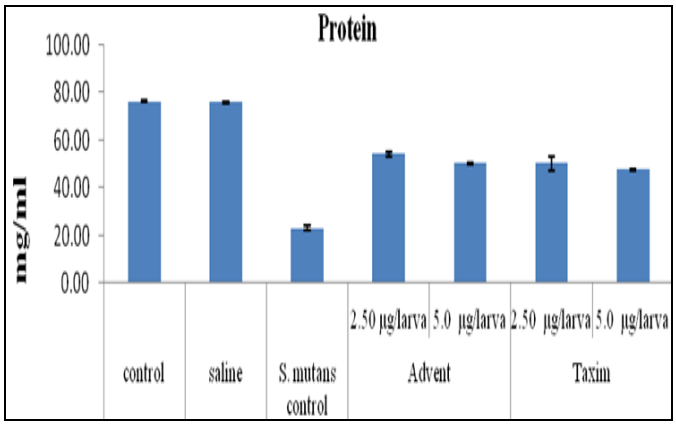


**FIG. 1: CHANGES IN THE HAEMOLYMPH PROTEIN DUE TO *S. MUTANS* INFECTION AND ANTIBIOTIC TREATMENT IN THE SILKWORM, *B. MORI* LARVA THROUGH THE HAEMOCOEL ROUTE**

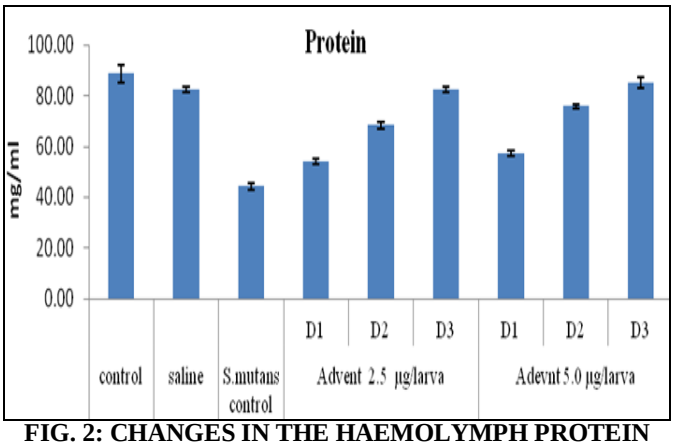


**FIG. 2: CHANGES IN THE HAEMOLYMPH PROTEIN CONTENTS OF *B. MORI* LARVA AS INFLUENCED BY *S. MUTANS* BACTERIA AND ADVENT ANTIBIOTIC ADMINISTERED THROUGH THE ORAL ROUTE**

Significant improvement was also observed in the protein content of the larval batches treated with Taxim antibiotic, but not to the extent of Advent. However, the highest improvement in the protein content measuring 79.17 mg/ml was recorded in the larval batches treated with three doses of 5.0 µg/larva/dose of taxim antibiotic.

Whereas, with the administration of a single and two doses of antibiotic, the amount of protein content recorded was 51.63 and 63.83 mg/ml respectively. However, 2.50 µg/larva/dose of antibiotic treatment, the highest content of 67.63 mg/ml protein was recorded from the three doses followed by two and a single dose that account to 51.23 and 43.43 mg/ml respectively, which is statistically significant at $p<0.05$ **Table 1**.

**Quantitative Changes in the trehalose Content of Haemolymph of the Larvae Infected with *S. mutans* and Treated with Antibiotics:**

**Administration of *S. mutans* and Antibiotics - Haemocoel Route:** In contrast with protein, the trehalose content was considerably increased to a record of 8.54 mg/ml after *S. mutans* infection from 5.33 mg/ml in the non-infected (control) larval batches. A remarkable decline in the trehalose content was observed that varied against the concentration of antibiotic administered to the larvae. Advent at the concentrations of 2.50 and 5.0 µg/larva treated larval batches exhibit 5.61 and 6.32 mg/ml of trehalose content respectively **Fig. 4**. Notably, a discrete difference was noticed between the two antibiotics, wherein the trehalose content was high in Taxim compared to Advent treated

batches. At the concentration of 2.50 and 5.0 µg/larva of taxim, 6.82 and 6.45 mg/ml of trehalose content, respectively was recorded, which is comparatively lesser than *S. mutans* infected larval batches **Fig. 4**. All these data are statistically significant at $p<0.05$ **Table 1**.

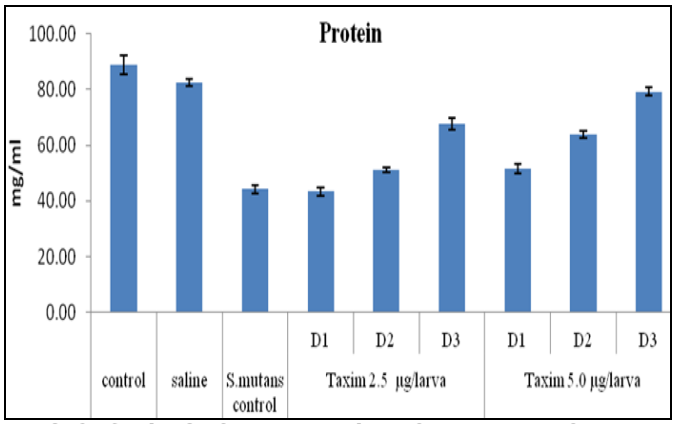


**FIG. 3: CHANGES IN THE HAEMOLYMPH PROTEIN CONTENTS OF *B. MORI* AS INFLUENCED BY *S. MUTANS* BACTERIA AND TAXIM ANTIBIOTIC ADMINISTERED THROUGH ORAL ROUTE**

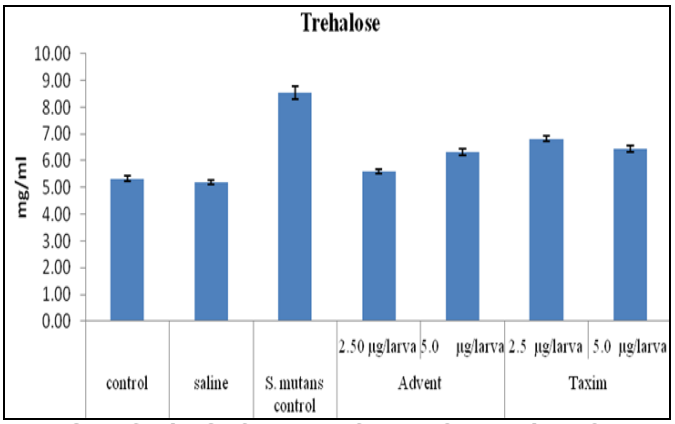


**FIG. 4: CHANGES IN THE SILKWORM HAEMO-LYMPH TREHALOSE CONTENT DUE TO *S. MUTANS* INFECTION AND ADMINISTRATION OF ANTI-BIOTICS THROUGH INTRA-HAEMOCOEL ROUTE**

Administration of *S. mutans* and antibiotics – oral route: *S. mutans* infection achieved through oral route has accelerated rise in the trehalose content to 11.51 mg/ml as against 6.97 mg/ml in the healthy larval batches. The antibiotic - Advent treatment boosted the silkworm health by declining the trehalose content to 9.59, 8.97 and 8.02 mg/ml in larval batches treated with a single, two and three doses of 2.50 µg/larva/dose antibiotic respectively as against bacteria-infected batches.

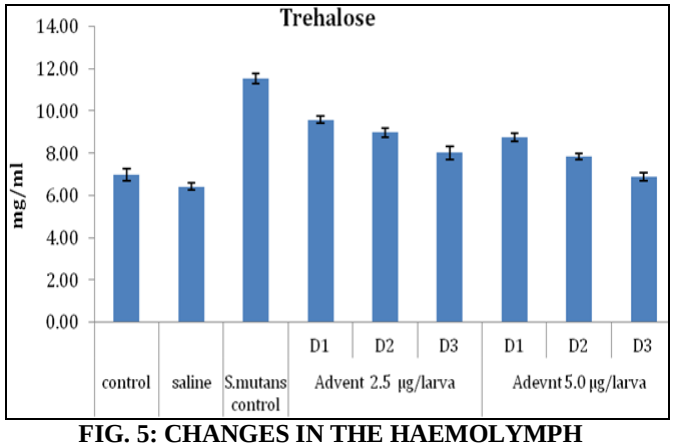


**FIG. 5: CHANGES IN THE HAEMOLYMPH TREHALOSE (MG/ML) CONTENT OF *B. MORI* AS INFLUENCED BY *S. MUTANS* BACTERIA AND ADVENT ANTIBIOTIC ADMINISTERED ORALLY**

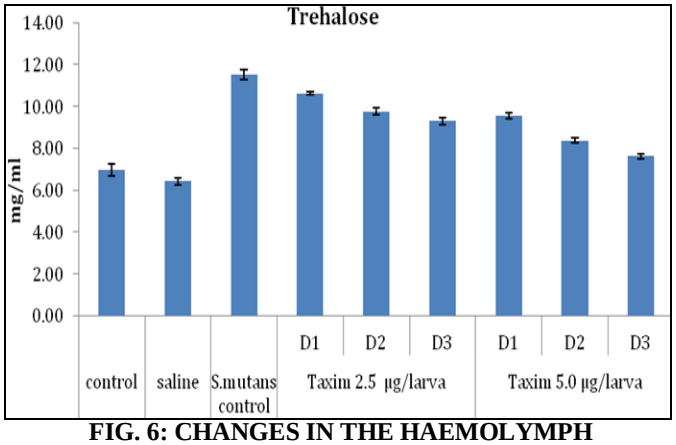


**FIG. 6: CHANGES IN THE HAEMOLYMPH TREHALOSE CONTENT OF *B. MORI* AS INFLUENCED BY *S. MUTANS* BACTERIA AND TAXIM ANTIBIOTIC ADMINISTERED THROUGH ORAL ROUTE**

**TABLE 1: STATISTICAL ANALYSIS OF THE DATA FOR THE CHANGES IN THE PROTEIN AND TREHALOSE COMPONENTS AS AFFECTED BY *STREPTOCOCCUS MUTANS* INFECTION AND ANTIBIOTICS ADMINISTERED THROUGH HAEMOCOEL AND PERORAL ROUTE**

| Route of administrations | Antibiotics | Protein | | Trehalose | |
|---|---|---|---|---|---|
| | | F-value | Significance($p \leq 0.05$) | F-value | Significance($p \leq 0.05$) |
| Haemocoel | Advent | 1345.51 | 0.000** | 435.67 | 0.000** |
| | Taxim | 1345.51 | 0.000** | 435.67 | 0.000** |
| Peroral | Advent | 523.028 | 0.000** | 289.927 | 0.000** |
| | Taxim | 527.968 | 0.000** | 475.055 | 0.000** |

As the concentration of the antibiotic-Advent increases to 5.0 µg/larva/dose, the trehalose content was further declined to 8.73, 7.83 and 6.87 mg/ml as a response to a single, two and three doses

respectively, one of which was equivalent to healthy larval batches **Fig. 5**. All these data are statistically significant at $p<0.05$ **Table 1**. Despite, the antibiotic - Taxim showed a good response that does not as effective as that of Advent. As a result, 10.62, 9.75 and 9.29 mg/ml of trehalose content was recorded after a single, two and three doses of 2.50 µg/larva/dose of Taxim administration to the *Streptococcus mutans* infected larvae respectively. Interestingly, as the concentration increased to 5.0 µg/larva/dose that substantially declined the trehalose content to 9.54, 8.37 and 7.63 mg/ml respectively **Fig. 6**.

**DISCUSSION:** It is a well-known fact that bacterial contagion manifests the whole-body protein homeostasis. But, the consequence of *S. mutans* infection on the host, *B. mori* protein synthesis and its recovery after antibiotic treatment has not been elucidated so far. Hence, the human dental caries (*S. mutans*) - silkworm disease model developed in our laboratory [10] was used to uncover a shift in protein and trehalose synthesis and accumulation in the hemolymph of the silkworm larvae as affected by *S. mutans* infection and clinically in practice antibiotics for the first time. Notably, *S. mutans* administered both hemocoel and oral routes depressed the synthesis of protein that accounts to 69.60 and 50.21% less over its respective healthy larval batches.

Contrastingly, trehalose content estimated by using the same sample of hemolymph from the same batches of silkworm larvae was found increased over 60.22 (haemocoel) and 65.13% (oral) in comparison with the healthy larvae. These biochemical data are well linked with the $LC_{50}$ values of *S. mutans* infection that induces 50% mortality as has been correlated with the reduction of chitin content in the cuticle of the *S. mutans* infected larvae [10] and substantiate that *S. mutans* - silkworm is a promising dental caries disease model for further investigation. Insects are depending on proteins for maintenance of its different physiological functions, development and metamorphosis [12]. The pathogenic infection induces significant biochemical and physiological modifications by depressing the synthesis of protein synthesis [18], which is in conformity with the present investigation due to *S. mutans* infection in the silkworm larvae of *B. mori*.

In addition, consumption of mulberry leaf plays a prominent role in the accumulation of hemolymph proteins through a high rate of conversion and assimilation [19]. Thus, it is also one of the important factors that affecting accumulation of proteins in the hemolymph of silkworm larvae infected with *S. mutans*, which exhibit a drastic reduction in consumption of mulberry leaves (data not presented). Generally, the hypertrehalosemia occurred for three main reasons *viz.*, flight, feeding, and parasitic infections [20, 21]. The increased amount of trehalose was due to multiplicities of infection [22]. The major source of trehalose in the hemolymph is due to the breakdown of glycogen in the fat body [23] or may be due to the release of trehalose as a result of histolysis of various tissues or the release of the fat body. Trehalose is a non-reducing disaccharide comprising two glucose molecules. It is the key sugar circulating in the hemolymph (blood) of silkworm.

The results of the present work strongly support the possibility of histolysis of the fat body due to pathogenic infection. Our inference is in agreement with the published work [24] wherein significant elevation of trehalose content in infected hemolymph reported is might be due to the conversion of glycogen into trehalose and its subsequent release into the hemolymph. In this way, the infection dependent disruption of fat bodies could be the cause of reduction in the protein content and also increased trehalose level in the hemolymph of *S. mutans* infected larvae compared to healthy larvae. Another reason could be that *S. mutans* must have utilized available hemolymph trehalose for deriving energy for the pathogenesis. These findings are the first and foremost basis of biochemical evidence to envisage successful growth and development of *S. mutans* in the body of silkworm larvae, which is a unique feature of the study.

As the level of trehalose rises, its synthesis in the fat body is inhibited and UDP-glucose is diverted to glycogen synthesis. Researchers were found that, in insects, injection of trehalose reduces the lipid concentration in hemolymph, implying that an inverse relationship exists between the hemolymph concentration of lipid and trehalose [25, 26]. Thus, the present findings further support the hypothesis that trehalose plays a pivotal role in response to several

biological functions as a physiological adaptation and an energy source in insects [27]. Clinically advocating antibiotics for human also have therapeutic effects in silkworm [11] and proved by administering the Amoxycillin antibiotic against *Bombyx mori* nuclear polyhedral virus [28]. In the light of these findings and importance, we have tested two commercially available antibiotics - Advent and Taxim that are clinically advocated for human dental caries pathogen *S. mutans* infection. Notably, the *S. mutans* infected silkworm larvae responded very well to these antibiotics and showed a clear difference in the recovery of protein synthesis and under-synthesis of trehalose as regulated by the antibiotics administered through haemocoel and oral routes for the first time.

Interestingly, the highest rate of protein synthesis was recorded with 133.81% in the larval batches treated with 2.50 µg/larva of Advent through hemocoel route indicate that the larvae have recovered from the *S. mutans* infection and switched over to synthesis of protein for normal growth and development. Concomitantly, the trehalose content which was overproduced (60.22%) might be due to breakdown of glycogen in the fat body [23] and/or due to the release of trehalose as a result of histolysis of various tissues of the silkworm larvae infected with *S. mutans.* It was reduced to 34.30% at the concentration of 2.50µg/larva/dose of Advent administered through haemocoel route. This clearly indicates that the antibiotics administered through the inter-venous (hemocoel) route was successfully inhibited the bacterial growth and facilitating the larvae for the normal synthesis of proteins that are essential for normal growth and development of the larvae and metamorphosis [12].

It is noteworthy to record that highest rate of protein synthesis was recorded after three doses of Advent and Taxim at the concentration of 5.0 µg/larva/dose administered to the *S. mutans* infected larvae that account to 92.62 and 78.93% respectively. Concomitantly, at the same dose and concentration of Advent and Taxim antibiotics influenced a reduction nearly 40.30 and 33.75% of trehalose content that has been over synthesized due to *S. mutans* infection (65.13% over healthy larvae) compared to control. The changes in the protein and trehalose contents clearly explicit that the larvae were successfully recovered from *S. mutans* infection and regain the cellular machinery in regulation of protein and trehalose synthesis, which again substantiate the observations recorded in the present study with respect to the inter-venous route of antibiotics administration.

**CONCLUSION:** Taken together, our results, aptly demonstrated the deleterious effect of *S. mutans* infection on the silkworm protein and trehalose contents and its regulation after antibiotic treatment, which is mode of treatment, concentration, dosage, and drug dependent. Thus, we propose a similar type of systematic studies in human suffering from dental caries and evaluate new drugs against *S. mutans* using a silkworm model system developed by our group before the pre-clinical trial.

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**CONFLICTS OF INTEREST:** Nil

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