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Human Dental Caries Pathogen *Streptococcus* Mutans affect Chitin Synthesis and that Restored by Antibiotics in the Silkworm, *Bombyx mori*

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Abstract
Human dental caries pathogen *Streptococcus mutans* induced silkworm (*Bombyx mori* L.) disease model was used to examine the bactericidal effect on the cuticle chitin synthesis and its restoration due to antibiotics - Advent and Taxim that are clinically advocated to cure pulpal infections caused due to dental caries. Experimental data of the present investigation revealed that *S. mutans* infection distorted the synthesis and precipitation of chitin in the silkworm cuticle. Compared to control batches, a reduction of 35.50 and 39.83% of chitin was noticed in the larvae inoculated *S. mutans* through haemocoel and peroral routes respectively. Interestingly, antibiotics administered through the same routes accelerated a remarkable recovery of 33.8 (haemocoel) and 65.25% (*per-os*) of chitin respectively for Advent, while it was 21.77 and 46.75% for Taxim antibiotics. Thus, it is proven for the first time that inoculation of *S. mutans* both the routes were successful by way of inhibiting chitin synthesis and prepetition in the cuticle of the larvae and restoration of chitin synthesis as a response to antibiotics differs between two antibiotics, which are concentration and dose-dependent.

Keywords
Antibiotics, *Bombyx mori*, chitin, cuticle, dental caries, *Streptococcus mutans.*

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1. INTRODUCTION
In recent years, silkworms (*Bombyx mori* L.) have extensively been in use to assess the pathogenicity of bacteria causing varied diseases in human [1-3] and proposed as an invertebrate model system due to its unique advantages over other experimental invertebrate model. Besides, the silkworm death, either genetic or imposed, does not involve any bioethical issues [4]. Additionally, most of the studies have confirmed that silkworms are highly sensitive to human pathogenic microorganisms, pathogenic fungi, antibiotics, and pesticides [5]. Similar to any other mammalian models, drugs and novel molecules can be administered both oral and intravenous routes [6, 7] to the silkworm larvae and different tissues/organs such as, cuticle (exo-skeleton), midgut, fat body and haemolymph shall be collected with ease for toxicity and efficacy testing. Hence, recently, the silkworm larvae are used as a model system for *in-vivo* testing of nanoparticles for

its toxicity and efficacy [7]. Considering the significance of silkworm as a model system for biomedical applications, the *Streptococcus mutans* causing dental caries, which further leads to pulp and periapical infection in human has been well established as disease model system in our laboratory that open ample scope for prospective investigations, hence this study.

In humans, dental caries is well known as one of the most common and expensive diseases of the world and has become a major challenge for the health service provider. To address this disease, which are a triad fermentable carbohydrates, sticky tooth surface and acid produced by microorganism like *S. mutans*, various simulation models have been developed that mimics *in-vivo* oral niches and habitats, but it is difficult to compare methods that use different adhesion surfaces [8]. So, *S. mutans* - silkworm disease model established in our laboratory shall be one of the best *in-vivo* models for human dental caries, but its impact on other tissues/organs of the silkworm larvae is enigmatic. Basically, the *S. mutans* has a simple metabolism through a chemical process that occurs in an organism to maintain its growth and development. *S. mutans* cleave or cut sucrose from the host's diet into glucose and fructose. The bacteria grow on these food particles with channels in their cell wall that help molecules cross the cell wall and get into the cell. In absence of it, the bacteria secrete an enzyme that breaks down certain tissues in its environment and establishes itself with the host, which is unclear with the silkworm that warranted systematic investigation. In clinical practices, a most promising approach for the control of *S. mutans* infection involves the use of antibiotics, chitosan-based mouthwash [9] and chitosan nanoparticles (CS-NPs) for sustained drug release [10, 11].

Chitosan is a derivative of chitin [12]. The chitin is a polysaccharide, main component of insect exoskeleton. Cuticle of the silkworm is a non-living matrix of carbohydrate and protein secreted by the underlying monolayer of epithelial cells that cover the quasi-totality of the surface of the insect [13]. Thus, it is considered as a mechanical barrier that effectively safeguards against microbial invasion [14, 15]. The major structural component of the cuticle is chitin-a polymer of N-acetyl-β-D-glucosamine [16]. The S.mutans use an inducible system for the metabolism of N-acetyl-glucosamine and glucosamine for its growth. A recent correlation that has been made between exoskeleton of insects and human epithelial tissue explicit that chitin mainly comprises a polymer chain of β-linked N-acetyl glucosamine [17] is responsible for newly forming human enamel [18]. Thus, we report here the impact of S. mutans on the components of silkworm cuticle and the action of clinically advocated antibiotics not only against bacterial infection but also restoration of cellular machinery for the synthesis of chitin in the silkworm larvae.

2. MATERIALS AND METHODS

2.1.1 Experimental animal

The larvae of *B. mori* strain NB_4D_2 were reared providing mulberry leaves in the laboratory following standard rearing procedures [19].

2.1.2 Source of Bacteria

Pure culture of *Streptococcus mutans* (MTCC 890) procured from Microbial Type Cell Culture and Gene bank, Chandigarh, India was used in the present investigation. The bacteriological media used in the study was Brain-Heart-Infusion (BHI) broth and agar was procured from HIMEDIA laboratories.

2.1.3 Antibiotics

Commercially available antibiotics advocated for odontogenic infections, injections Advent, *i.v.* (Amoxicillin + potassium clavulanate) manufactured by CIPLA LIMITED and Inj. Taxim, *i.v/i.m.* (Cefatoxin) manufactured by ALKEM HEALTH SCIENCE was purchased from the registered local pharmacy.

2.2 Preparation of bacterial sample

According to the supplier's protocol, the lyophilized cells of *S. mutans* were rejuvenated. For colony development, 20 µL of the rejuvenated sample was cultured on BHI agar by incubating at 37° C for 48 hr. Manual colony counter was used to obtain 210 bacterial colonies. A single colony picked with the help of a sterile loop was re-suspended in BHI broth to acquire 2.1 x 10^{10} CFU/mL) and considered as bacterial stock suspension. Further dilution was performed to derive varied quantum of bacterial cells based on the optical density at 600 nm (Elico SA 165, India) using 0.5 McFarland (1.5 x 108 CFU/mL) as standard and incubated for 12 hr. The viable cells from each concentration were isolated by adding required quantum of 0.3% normal saline and centrifuged at 4000 rpm for 10 min at 4° C. Bacterial cells isolated were re-suspended in 10 x Phosphate Buffer Solution to derive the required density of bacterial suspension in each of serial concentration for further experiments.

2.3 Bacterial infection and antibiotics treatment

For the intravenous test, a bacterial suspension of LC_{50} was injected into haemocoel (dorsal blood vessel) of healthy fifth instar larvae on day-3 (detail procedure has not been presented here since it is under the process of a patent). Devoid of bacterial inoculation, absolute control and a positive control batch that received the same amount of PBS was maintained. After initial inoculation of bacterial suspension with a lag period of 12 hr, antibiotics - Advent and Taxim at the concentrations of 2.50 and 5.0 µg in sterile water (as per the manufacturer instructions) were injected into different groups of larvae. All the treated and control batches of larvae were fed with fresh mulberry leaves until spinning of cocoons.

For the per-oral test, *S. mutans* bacterial suspension of LC_{50} was smeared on to a unit quantity of fresh mulberry leaves and fed to the larvae maintained in replicates with ten larvae each (detail procedure is protected in lieu of patent). Untreated leaves were fed to the control group after the fourth moult. The same volume of antibiotic suspension at the concentrations of 2.50 and 5.0 µg of Advent and Taxim was smeared on the same quantum of mulberry leaves and fed larvae after 12 hr of the lag period, which forms the first dose. On 3rd and 4th day of the fifth instar, 2nd and 3rd dose of antibiotic-treated leaves for different test batches and untreated leaves for the control group were provided. All the groups of the larvae were maintained in the same environment employing standard rearing procedures [19].

2.4 Analysis of cuticle chitin content in the *S. mutans* infected and antibiotics treated silkworm larvae

Fifth instar 5 days old silkworm larvae were randomly collected from all the groups to determine chitin content in the cuticle [20]. Briefly, the larvae selected from different treatment groups were dissected separately after treating them in boiling water for about 1 min to obtain pure tissue of cuticle devoid of other tissues associated with it. After washing the cuticle with distilled water, initial weight was recorded that denote as 'm0' and after drying at 96-98 °C its weight was recorded as 'm1'. The difference between m0 and m1 shall be the final weight of cuticle. The resultant cuticle was soaked in Ether for 10–15 min and the Ether was replaced twice to remove the crude fat during the process. After washing in distilled water followed by drying at 96–98 °C the weight of the cuticle was recorded as 'm2'. The difference between m1 and m2 was the weight of crude fat. Further, the cuticle (m2) was placed in the 18% KOH solution for 6 h at 60 °C and KOH solution was replaced four times during this process to remove protein. Then the cuticle was washed thrice in Ethanol followed by distilled water and dried at 96–98 °C and its weight was recorded as 'm3'. The difference between m2 and m3 was the weight of protein. The resultant cuticle was collected in a high heat resistant Quartz crucible and then it was placed in a muffle furnace for 3 h at 550 °C and its weight was recorded 'm4'. The difference between m3 and m4 shall be the weight of chitin, while m4 and m0 was the weight of the ash.

3. RESULTS

The human dental caries pathogen *S. mutans* exhibit characteristic bacterial symptoms for both *per-os* and haemocoel routes of administrations indicating its successful growth in the body of silkworm larvae. Confirming its pathological symptoms, commercially available antibiotics – Advent and Taxim of varied concentration and dosage administered to the larvae has shown substantial recovery from bacterial infection. Thus, cuticles of all the treated groups collected were subjected for analysis to evaluate *S. mutans* growth by metabolizing n-acetyl glucose amine which is a biopolymer of chitin [21] while such information is lacking in *B. mori*.

3.1 Administration of *S. mutans* and antibiotics - haemocoel route

Interestingly, *S. mutans* injected into the larvae through haemocoel route has shown successful pathogenesis. As a result, there was a drastic reduction in the cuticle chitin content measuring 31.84% in the infected group, while it was 49.37% in the non-infected batches that indicate nearly 35.50% of chitin content was affected by *S. mutans* infection. Concurrently, the antibiotics conquered the activity of *S. mutans* and enabled restoration of chitin synthesis in the cuticle. Consequentially, the content of chitin recorded was 42.61 and 40.84% in the cuticle of larvae treated with 2.50 and 5.0 µg/larva of Advent that denote an improvement of 33.81 and 28.25% respectively from that of *S. mutans* infected batches (31.84%). On the other hand, 35.83 and 38.78% of chitin was recorded in the larvae treated with the same concentration of Taxim that shows 12.52 and 21.77% of higher chitin content as influenced by antibiotic than infected batches respectively (Fig. 1). All these data are statistically significant at $p<0.05$ (Table 1).

3.2 Administration of *S. mutans* and antibiotics - Peroral route

To examine the dose-dependent effect of antibiotics against *S. mutans*, the present study was further extended by using a single, two and three dosages of antibiotics separately in parallel at appropriate duration. Due to the limitation in injecting the pathogen and antibiotics frequently into haemocoel and to avoid multiple injuries to the larvae, we have modified the strategy and administered the *S. mutans* and antibiotics per-orally. Interestingly, the bacteria inoculated to the larvae per-orally were also exhibited characteristic bacterial disease symptoms. Upon confirmation of the infection through typical bacterial disease symptoms, three doses of antibiotics were administered through mulberry leaves at the stipulated period as noted in the materials and methods section.

Notably, the chitin content recorded from *S. mutans* infected batches of the larvae was 29.44%, which is lesser than the control batches (48.94%) and there was a drastic decline in the chitin content of 39.83%, which is statistically significant at $p<0.05$. After antibiotic treatment, the highest chitin content of 43.77% was recorded as a response to three doses, while it was 32.84 and 35.61% respectively for single and two doses of Advent at the concentration of 2.50 µg/larva/dose. Interestingly, as the concentration of Antibiotic-Advent increases to 5.0 µg/larva/dose, the chitin contents were also found elevated to 48.65% that is equivalent to normal control (48.94%) and thereby there was a higher rate of recovery of 65.25% in the chitin content due to antibiotic treatment from the *S. mutans* infected batches (29.44%, Fig. 2). In case of two doses of antibiotic - Advent treatment, it was 41.02% that eventually less by measuring 37.96% chitin content for a single dose of antibiotic treatment, which is higher than that of *S. mutans* infected larval batches (29.44%) as statistically significant at $p<0.05$ (Table 1).

Contrastingly, the highest of 43.20% of chitin was recorded from the larval batches that received three doses of Taxim at the concentration of 5.0 µg/larva/dose (Fig. 3). Though it is slightly lesser than the normal control (48.94%), but there was an improvement of 46.75% as against *S. mutans* infected larval batches (29.44%) that statistically significant at $p<0.05$ (Table 1). At the same concentration, chitin content recorded 39.22% was from the larval batches that received two doses followed by 33.16% for a single dose of antibiotic-Taxim. As the concentration of antibiotic reduces to 2.50 µg/larva/dose, again, higher chitin content of 37.19% was recorded as a response to three doses of antibiotic-Taxim, followed by 32.71 and 30.67% respectively for two and single doses of Taxim antibiotic. So, while the chitin content in the cuticle of *S. mutans* infected larvae declined, after antibiotic - Taxim peroraly administered successfully inhibit the bacterial infection. Concomitantly, the host cellular machinery accelerated the synthesis of chitin on a dose-dependent manner to the extent of 46.75% for three doses of Taxim (43.20%) as against *S. mutans* control (29.44%) but does not elicit to the extent of normal control (48.94%, Fig. 3). All these data are statistically significant at $p<0.05$ (Table 1).

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

Route of administrations	Antibiotics	Chitin(%)		Protein(%)		Ash(%)	
		F-value	Significance ($p \le 0.05$)	F-value	Significance ($p \le 0.05$)	F-value	Significance ($p \le 0.05$)
Haemocoel	Advent	110.401	0.000**	20.106	0.000**	31.179	0.000**
	Taxim	110.401	0.000**	20.106	0.000**	31.179	0.000**
oral	Advent	51.022	0.000**	23.528	0.000**	16.561	0.000**
	Taxim	448.957	0.000**	97.308	0.000**	17.654	0.000**

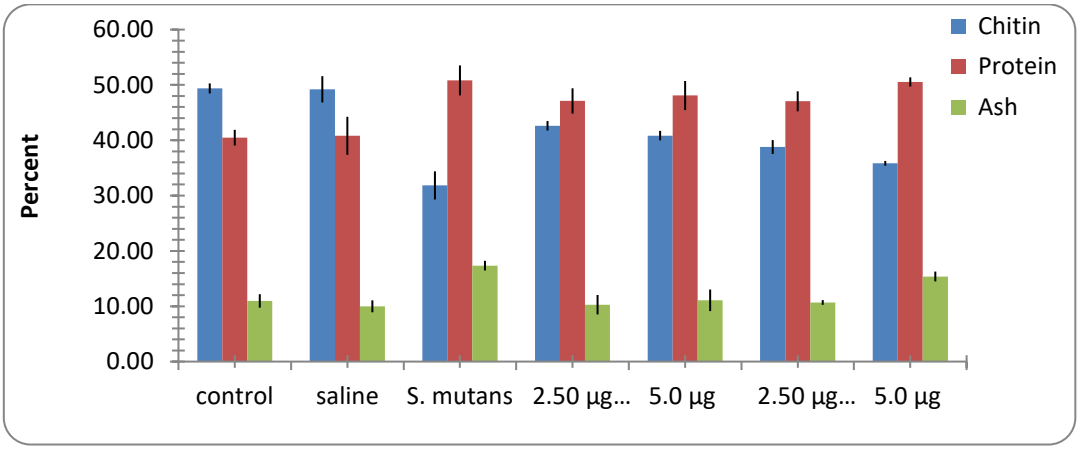

Figure 1. Changes in the cuticle components as affected by *Streptococcus mutans* infection and antibiotics administered through haemocoel route.

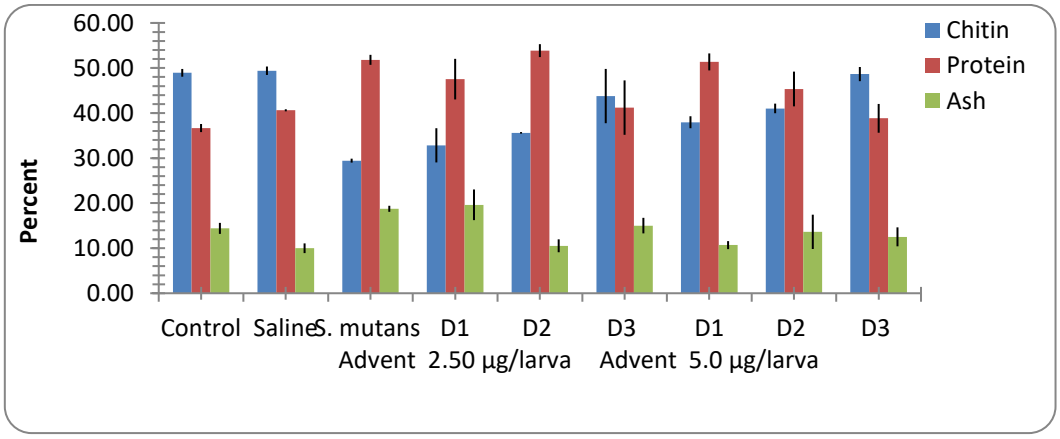

Figure 2. Changes in the cuticle components as affected by *Streptococcus mutans* infection and antibiotic – Advent administered peroral route.

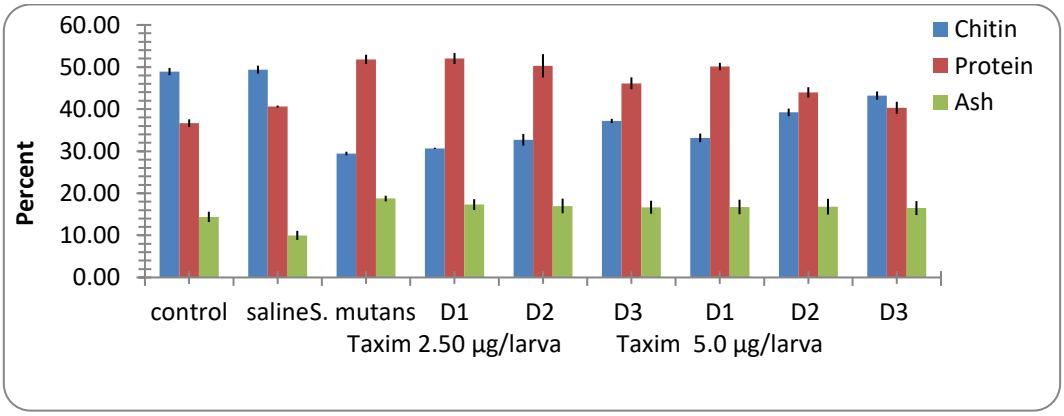

Figure 3. Changes in the cuticle components as affected by *Streptococcus mutans* infection and antibiotic – Taxim administered peroral route.

4. Discussion

For the first time, *S. mutans*, a human dental caries pathogen has shown successful pathogenesis for both *per-os* and haemocoel routes of inoculation in the silkworm, *B. mori* larvae with characteristic bacterial symptoms. Thus, it is noteworthy to

mention that the silkworm, *B. mori* larvae shall be a promising human dental caries pathogen *S. mutans* disease model for evaluation of any new medicinally important compounds or drugs as has been previously demonstrated for *B. mori* nuclear polyhedrosis [22].

Cuticle of the *S. mutans* infected larva when subjected for the biochemical analysis, there was ~31.84% decline in the content of chitin in comparison with the healthy larvae, which is near equivalent to LC_{50} value of bacteria that induce 50% larval mortality. In insects, the cuticle is non-living matrix of carbohydrate and protein synthesised by epithelial cells [13] and acts as a mechanical barrier to safeguard microbial invasion [14,15]. However, the main structural component of insect exoskeleton (cuticle) is chitin, a polymer of N-acetyl-β-D-glucosamine [16, 17]. An interesting feature of the present investigation is, such that *S. mutans* use the inducible system for the metabolism of *N*-acetyl glucosamine and glucosamine. As a result, *S. mutans* has grown successfully in the body of the silkworm larvae by metabolizing n-acetylglucose amine [21] that resulted in a drastic decline in the content of chitin in the cuticle of infected larvae compared to healthy larvae of *B. mori*.

These data precisely indicate that *S. mutans* being highly acidogenic produces short-chain acids, which softens the hard tissue like cuticle in the silkworm larvae as in the case of the human tooth. In support of this, recent study has made a correlation between exoskeleton of insects and human epithelial tissue, which encompasses a polymer chain of β-linked N-acetyl glucosamine [17] and monomeric unit of this, is one of the components of newly forming human enamel [18]. And hence, the organic content is well correlated with human dental caries and degradation of chitin content in the cuticle of the silkworm larvae as reported in the present study. Besides, isozymes of glucosyltransferases, catalyze and metabolize sucrose to synthesize insoluble extracellular polysaccharides, which increase bacterial adherence to the tooth surface and persuade biofilm formation [23].

Notably, when the antibiotics - Advent and Taxim, which are routinely used in clinical practices for treating pulpal infection, administered through dorsal blood vessel and peroral, boosted the silkworm larval growth, probably by inhibiting the bacterial cell wall synthesis that leads to bacterial death [24]. Besides, the host cells were restored its normal synthetic machinery to synthesise chitin in the cuticle of the larvae, as a result, there was an elevation in the amount of chitin content in antibiotics treated larvae. However, we have noticed a distinct difference between the two antibiotics in its action against *S. mutans* and improvement of chitin content in the silkworm larvae, which are absolutely dose, concentration and drug dependent. Thus, antibiotics–Advent and Taxim clinically advocated to cure *S. mutans* infection is well-substantiated *in-vivo* for the first-time using silkworm - *S. mutans* disease model.

Contrastingly, chitin present in the insect's cuticle, including silkworm as observed in the present study, is known to have a polymer – chitosan, which can be obtained by alkaline hydrolysis [25]. Chitin and Chitosan are known to have antimicrobial potential, which has been investigated against a wide range of organisms through *in-vitro* and *in-vivo* experiments [26]. Surprisingly, a reduction in the chitin content of silkworm larval cuticle despite observed due to *S. mutans* infection, but the action of chitosan to govern the bacterial infection remain obscure and offers detailed investigation to uncover the molecular mechanism underlies in this phenomenon. Chitosan and its nanoparticles have potential application in the field of pharmaceutical, food, agriculture, textile, and tissue engineering industry, because of its biocompatibility, biodegradability and lack of toxicity [27]. Hence, chitosan-based mouthwash was developed, which has significantly higher antibacterial activity against *Streptococcus* and *Enterococcus* species than commercial essential oils and chlorhexidine mouthwashes [9]. Later, chitosan nanoparticles (CS-NPs) have been developed for sustained drug release [10, 11].

With these advances, we propose two novel approaches (a) large scale production of CS-NPs utilising either silkworm larval or pupal cuticles, which are abundantly available as by-products of sericulture industry and (b) *in-vivo* evaluation of specially formulated dental varnishes, mouthwashes and drugs against *S. mutans* using silkworm - dental caries disease model developed in our laboratory, which has a great value in predicting potential caries-preventive strategies.

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