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Role of microorganisms in the gut of silkworms

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Abstract
The major role of the microbiome presents in the digestive tract of silkworm involved in digestion and absorption process by producing cellulose, xylan, pectin and starch degrading enzymes. Its function is peaked at based on pH levels of the digestive juice to the respective enzymes. In addition, gut microbiota of silkworm was involved in the different host immune responses to different microbial infections by producing the antimicrobial peptides and immune signaling pathways. Silkworm and their core intestinal microbes established a symbiotic relationship of co-adaptation and co-evolution to maintain survival and reproduction in the evolutionary process.

Keywords: Silkworm gut, microbiome, host metabolism, defensive mechanisms

Introduction
Insect guts present distinctive environments for microbial colonization, and bacteria in the gut potentially provide many beneficial services to their hosts. Insects display a wide range in degree of dependence on gut bacteria for basic functions. Most insect guts contain relatively few microbial species as compared to mammalian guts, but some insects harbor large gut communities of specialized bacteria (Engel and Moran, 2013) [7]. The diversification and evolutionary success of insects have depended in part on their myriad relationships with beneficial microorganisms, which are known to upgrade nutrient-poor diets; aid digestion of recalcitrant food components; protect from predators, parasites, and pathogens; contribute to inter and intraspecific communication. Some insect species provide useful laboratory models for experimental work on microbial communities and their interactions with hosts, particularly for the understanding of immunity and metabolic interactions (Lemaitre & Hoffmann, 2007).
Microorganisms may produce some of the digestive enzymes to provide essential nutrients or assist in important biochemical function related to host food ingestion (Mckillip *et al.*, 1997; Broderick *et al.*, 2004; Pandiarajan, 2015) [31, 4, 35]. The gut microflora of *B. mori* are associated with various physiological processes besides providing colonization resistance to invading pathogens through production of antimicrobial compounds.
In recent years attempts have been made in sericulture with nutrients such as proteins, carbohydrates, amino acids, vitamins, sterols, hormones, antibiotics etc., for better performance and get higher yield with quantity and quality cocoon (Sannappa *et al*., 2002) [4]. Oral administration of foliage of mulberry and eri silkworm supplemented with cyanobacteria, enhanced larval and shell weight subsequently commercial characters of cocoon (Kumar *et al*., 2009; Masthan *et al*., 2011) [18, 30].
Nutrition plays an important role in improving the growth and development of *Bomby mori*. The silkworm larva consumes all kinds of nutrients from the mulberry leaves to build its body and spin cocoon. The healthy growth of the silkworm and ultimately the economic traits such as, cocoon and grainage parameters are influenced largely by the nutritional status of the leaves fed to worms. Fortification of mulberry leaves to rear the silkworms is a useful modern technique to increase economic value of cocoon (Masthan *et al*., 2011) [30].

Predominant microorganisms present in silkworm gut
Gram positive
- *Bacillus circulans*
- *Bacillus subtilis*
- *Enterococcus* sp.
- *Staphylococcus aureus*

Gram negative

- *Aeromonas* sp.
- *Citrobacter freundii*
- *Escherichia coli*
- *Enterobacter* sp.
- *Klebsiella pneumoniae*
- *Serratia liquefaciens*
- *Proteus vulgaris*

The study taken to investigate the composition and diversity of gut microbiota in the 5th instar of silkworm larvae. Intestinal contents were collected at different hours in the 5th instar. The dominated phyla in the gut of larvae are *Firmicutes*, *Cyanobacteria*, *Proteobacteria*, *Actinobacteria* and *Bacteroidetes* were isolated and the dominated genera are *Enterococcus*, *Staphylococcus*, *Arthrobacter*, *Pseudomonas*, *Lactobacillus*, *Bacteroides*, *Paenibacillus* and *Serratia* were isolated respectively. Among these bacteria, the abundance of Gram-positive bacteria *Enterococcus* and *Staphylococcus* were present highest in the 5th instar larvae gut Hou *et al*., (2018) [13].

Changes in microbial population in silkworm gut

Artificial diets for silkworms had many potential applications and they are important role in sericulture. Dong *et al.,* (2018) [16] reported that differences between the gut microbiota of 5th-instar larvae reared on mulberry leaves and an artificial diet. The results revealed that the phylum level, *Cyanobacteria*, *Firmicutes*, *Proteobacteria*, *Bacteroidetes* and *Actinobacteria* are the dominant bacteria in the intestines of silkworm larvae of all the strains. But the abundance of dominant bacteria in the gut microbiota differed between the silkworm strains that were reared on mulberry leaves and artificial diet. The gut microbiota diversity lowered in the silkworm strains that were reared on the artificial diet. When the silkworm diet changed from mulberry leaves to the artificial diet, changes in gut microbiota in the silkworms affected host nutrient metabolism and immune resistance. The intestinal content of 6th day of fifth instar *B. mandarina* and *B. mori* larvae were analyzed. The predominant bacteria of phylum *Firmicutes* in *B. mandarina* (81.40%) and *B. mori* (81.85%) were identified. In *Firmicutes*, abundance of predominant bacterial genus *Enterococcus* in *B. mandarina* were comparatively higher than in *B. mori*. The genus *Advenella* belongs to phylum *Proteobacteria* were recorded only in *B. mandarina*. In addition to that the abundance of Unclassified_*Peptostreptococcaceae*, *Methanobrevibacter*, *Ignatzschineria*, *Petrimonas* and *Proteiniphilum* in *B. mandarina* were present and these bacterial genera were not detected in *B. mori* (Kumar *et al.,* 2019) [19].

The intestinal microbes of silkworm are abundant, including *Enterococcus, Delftia, Pelomonas, Ralstonia, Staphylococcus, Bacillus, Arcobacter*, etc. However, silkworm intestinal bacterial communities were also affected by forages, developmental stages, and gender (Liang *et al*., 2014; Sun *et al*., 2016) [23, 44]. More importantly, the silkworm intestinal microbes were significantly changed after pathogen infection. *Advenella* is a bacterial genus with high ability to degrade tetracycline (Zhang *et al*., 2015) [53-55]. The proportion of genus *Advenella* in *B. mandarina* gut were 11.54%, however, the proportion was lower than 0.01% in *B. mori*. *Advenella* is not only resistant to tetracycline but also to other chemical compounds (Caracciolo *et al*., 2010) [5]. The strong degradation ability of *B. mandarina* not only endanger mulberry but also harm to the other plants, suggested that higher abundance of *Advenella* bacteria in *B. mandarina* silkworm intestinal flora were involved in the decomposition of the toxic compounds of food plants (Kumar *et al.,* 2019) [19].

Synthesis of enzymes in the gut of silkworm

Microorganisms present in the silkworm gut are involved in the different enzyme production for digestion. They secrete the cellulose, starch, protein, pectin, xylan, lipids and fatty acids degrading enzymes.

Exploring bacterial populations with cellulolytic activity from the midgut of *B. mori* were proposed by Revathy and Pandiarajan, (2019) [38]. Cellulose degrading bacteria were harvested from the midgut of *B. mori.* All the gut isolates were subjected to plate assay separately, to display the efficient cellulose fabricator. Among them *Bacillus aryabhattai* and *Bacillus sp* has showed the highest level of cellulolytic activity. The Productivity of cellulase in *Bacillus aryabhattai* were detected and optimized at different pH, temperature, incubation time and different concentration of cellulose.

Anand *et al.,* (2010) [2] Eleven isolates were obtained from the digestive tract of *B. mori*, including the Gram positive *Bacillus circulans* and Gram negative *P. vulgaris, K. pneumoniae, E. coli, C. freundii, Serratia liquefaciens, Enterobacter* sp., *P. fluorescens, P. aeruginosa, Aeromonas* sp., and *Erwinia* sp.. Three of these isolates, *P. vulgaris, K. pneumoniae, C. freundii*, were cellulolytic and xylanolytic, *P. fluorescens* and *Erwinia* sp., were pectinolytic and *K. pneumoniae* degraded starch. *Aeromonas sp.* was able to utilize the corboxymethylcellulose and xylan. *S. liquefaciens* was able to utilize three polysaccharides including corboxymethylcellulose, xylan and pectin. *B. circulans* was able to utilize all four polysaccharides with different efficacy. *B. circulans, P. vulgaris, E. coli*, and *C. freundii* can produce digestive enzymes to help degrade carbohydrates, and alkaliphilic bacteria found and isolated from *B. mori* intestines can degrade polysaccharides, while the number of cellulose-decomposing bacteria in the intestines increases with increasing larval age (Anand *et al.,* 2010) [2].

The isolates from silkworm gut can produce cellulose and starch degrading enzymes in the traditional rearing way were *Alternaria sp. Preussia sp* and *Coprinellus radians.* Meanwhile, in the bioregenerative life support system were found *Enterococcus, Erwinia* and *Pantoea* can produce cellulase and amylase. The dominant populations of isolates may used to make probiotic products for nutrient absorption and disease prevention in bioregenerative life support system to improve gut microecology, yield and quality of animal protein (Xue *et al.,* 2015) [51]. *Alternaria* were able to use carboxymethylcellulose as substrates. It is reasonable that the function of *Alternaria* in the silkworm gut is associated with nutrient and energy digestion to ensure the normal growth (Deng *et al.,* 2014) [6]. *Aeromonas sp*. with xylanase activity was isolated from the intestine of the herbivorous insect, *Samia cynthia pryeri* (Roy *et al*., 2003) [39]. *P. vulgaris, C. freundii, S. liquefaciens* and *Klebsiella sp.,* were reported to be cellulose degrading bacteria and xyalnolytic bacteria (Alwin and Sripathi 2004).

The proteases secreted by *Stenotrophomonas maltophilia* can improve the digestion and absorption of mulberry leaves in *B.*

mori intestines (Wang *et al.*, 2016) [48]. Also, it has been reported that the abundance of lipase-producing bacteria in the gut microbiota of silkworms reared on an artificial diet changed significantly (Feng *et al.*, 2011) [10]. The lepidopteron muga silkworm larvae containing the gut isolate, *Bacillus* species was an effective player in digestion enhancement in by complementing the digestive enzyme and for improvement of host nutrition by providing vitamins and amino acids (Gandotra *et al.*, 2018) [11]. Although, Singh *et al.*, (2005) [42] observed improvement in larval body weight, cocoon weight, shell weight and pupation percentage of silkworm larvae when fed on mulberry leaves treated with a commercial probiotic formulation containing *Lactobacillus plantarum.* However, the extent of colonization of *L. plantarum* in the gut of silkworm is to be ascertained by using gnobiotic strains.

Mala and Vijila (2018) [29] reported that rearing and economic parameters of double hybrid silkworm race (CSR6 x CSR26) x (CSR2 x CSR27) fed on mulberry leaves fortified with *Bacillus licheniformis strain BMGB42* and *Bacillus niabensis strain BMGB17* individually and in different concentrations of combination. Among, these different treatments *Bacillus licheniformis* followed by *Bacillus licheniformis* + *Bacillus niabensis* (10^6cfu/ml) recorded maximum larval weight, effective rate rearing, cocoon weight, shell weight, pupal weight, shell ratio, silk productivity and filament length besides reduced larval mortality due to disease incidence and finer denier compared to control. The results showed that the *Bacillus licheniformis* can be used as probiotics in commercial silkworm rearing.

Enterococcus mundtii EMB156, isolated from the larval gut of the model organism *B. mori* efficiently produces lactic acid an important metabolite for industrial production of bioplastic materials (Liang *et al.*, 2018) [24]. The dominant gut-bacteria identified in muga silkworm *Antheraea assamensis* are *Bacillus* sp., *Proteus* sp., *Escherichia coli etc. In-vitro* screening of the isolates were done by amylase, pectinase, xylanase, lipase and cellulase test. The positive results revealed the possible influence of gut-bacteria on digestion and utilization of leaf carbohydrates, lipids and fatty acids for better productivity of muga silk (Bhuyan *et al.*, 2014) [3]. Study on *B. mori* has revealed that the abundance of *Cyanobacteria* in the intestines of silkworms reared on mulberry leaves may be related to the digestion of chlorophyll and other ingredients that are abundant in fresh mulberry leaves (Sun *et al.*, 2016) [44].

Diseases management using probiotics in silkworm

The gut probiotics are involved in the digestive utilization of feeds and detoxification of metabolite, stimulation of non-specific immune system. They also promote the production of vitamins and increase host resistance and compete with pathogenic bacteria by producing organic and antibiotic substance. Probiotics or bio remediators are gaining more popularity as eco-friendly supplementary feed to *B. mori*. The FAO/WHO (2001) [9] defines probiotics as 'Live micro-organisms which when administered in adequate amounts confer a health benefit on the host'. Digestion, absorption and disease prevention of silkworm were closely related to the microbiota lived in the silkworm digestive tract (Yuan *et al*., 2006) [52]. Impact of probiotics (*Lactobacillus*, *Saccharomyces cerevisiae* and effective microorganisms) treatment on mulberry leaves to modulate the economic parameters of 5th instar larvae of *B. mori* (Jeyapaul *et al*., 2004).

Probiotic application of *Streptomyces noursei* an actinomycete isolated from silkworm on the endogenous gut microflora and its antimicrobial activity. Probiotic applications of *S. noursei* have resulted in increase of endogenous actinomycetes population by 123.08 and 141.86 per cent, respectively in PM and CSR2. *In vitro* inhibition assays have shown that culture filtrates of *S. noursei* were having strong antibacterial activity against a wide range of gram positive and gram negative bacteria. Application of probiotics has paved way for eco-friendly management of silkworm disease management (Subramanian *et al.*, 2009) [43]. *S. noursei* was isolated and purified from the guts of Indian silkworm breeds. Functional analysis of *S.noursei* isolated from silkworm breeds revealed their antibiotic potential against a range of Gram positive and Gram negative bacteria and it was found to inhibit the germination of conidia of entomopathogens *B. bassiana* and *M. anisopliae in vitro* (Mohanraj, 2007) [32].

Application of probiotic microorganism *S. cerevisiae* increased the activities of enzymes such as amylase and invertase in the digestive the juice of silkworm which leads to enhance the immunity and quality of silk production (Esaivani *et al.*, 2014) [8].

Gloverin is one of the glycine rich antimicrobial peptide exclusively found in Lepidoptera insects. It is generally activated through the innate immune system in insects. The results showed that, recombinant Gloverin2 from *Bombyx mori* (BmGlv2) were synthesized using a prokaryotic expression system. Antimicrobial activity analysis revealed that BmGlv2 significantly inhibited the growth of gram-negative bacteria, *Escherichia coli* JM109 and *Pseudomonas putida*, by disrupting cell integrity (Wang *et al.*, 2018) [37]. Studies in silkworms reported that the abundance of *Enterococcus* in the intestines of silkworms reared on mulberry leaves is associated with increased immunity (Sun *et al.*, 2016) [44]. Lactobacillus 11/ 19-B1 enhanced the survival rate of larvae infected with *P. aeruginosa* by activating innate immunity in *B. mori* (Nishida *et al.*, 2016) [34].

E. coli and *B. bassiana* affect the immune response in silkworm intestines by increasing the expression of the signal-transduction-mediating transmembrane protein BmToll9 during the innate immune response (Wu *et al.*, 2010) [49]. These results indicate that different bacteria induce different immune responses in *B. mori*. *Lactobacillus* plays role in defense against pathogenic invaders of the wild silkworm *B. mandarina*. The intestinal floras of the silkworm and other wild insects have some resemblances and differences (Kumar *et al.*, 2019) [19].

Francisella tularensis established a symbiosis with silkworms, and bacteria were observed in the hemolymph. After infection with *F. tularensis*, the induction of melanization and nodulation, which are immune responses to bacterial infection, were inhibited in silkworms. Pre-inoculation of silkworms with *F. tularensis* enhanced the expression of antimicrobial peptides and resistance to infection by pathogenic bacteria. These results suggest that silkworms acquire host resistance via their symbiosis with *F. tularensis*, which may have important fitness benefits in natural reservoirs (Suzuki *et al.*, 2016) [46].

Enterococcus is a high frequency microorganism and can reduce pH of digestive juice and inhibit pathogens growth in intestinal environment through its products in silkworm. The

functions of *Enterococcus* complement each other. Its antibacterial function is enhanced in the acidic condition, at the same time the pH abatement of intestinal digestive juice can protect hosts against attacks of toxins. The appropriate supplementary of *E. faecalis* in diets can increase immunity of organisms (Shi *et al.*, 2015).

Expression of immune system against bacterial infection in silkworm

In contrast to our understanding of immune mechanisms in the fruit fly, those of the silkworm remain unclear. However, although the silkworm IMD, Toll, and JAK/STAT pathways are yet to be described, many laboratories have been working on the defensive responses of silkworms to infections by bacteria, viruses, fungi, and microsporidia, particularly since the sequencing of the silkworm genome.

Peptidoglycons (PGs) from *E. coli* and *Micrococcus luteus* are able to induce expression of certain antibacterial genes in silkworms, such as *cecropin B* and *lebocin 3* (Ha Lee *et al*., 20 2007; Tanaka *et al*., 2009) [12, 47]. *E. coli* and *B. subtilis* induce fat body-specific expression of Defensin B in the silkworm (Kaneko *et al*., 2008) [17], and expression of Attacin, Cecropin, Defensin, Gloverin, Lebocin, and Moricin is induced in the fat body in response to *P. aeruginosa*.

E. coli induces Dual oxidases (*Duox*) expression in the silkworm midgut, and larvae carrying a *Duox* deletion are more susceptible to bacterial infection (Hu *et al.*, 2013) [14]. Concentrations of H_2O_2 and NO in the silkworm gut significantly increase upon *P. aeruginosa* and *B. bombyseptieus* infection, and increased ROS levels can inhibit growth of these bacteria at the early stage of infection (Zhang and Lu, 2015; Zhang *et al*., 2015) [53-55]. The gram-positive bacterium *B. bombyseptieus* also activates AMP signaling, upregulating Attacin, Lebocin, Enbocin (which belongs to the cecropin family), Gloverin, and Moricin in the silkworm gut (Huang *et al*., 2009) [15].

Expression of immune system against fungal infection in silkworm

The Janus Kinase and Signal Transducer and Activator of Transcription (JNK/STAT) pathway is also involved in immune responses to fungal infection. *B. mori* C-type lectin 5 (BmCTL5) may function as a receptor that activates this pathway to defend against fungal but not bacterial or viral infection. It has also been confirmed that Cecropin A, Defensin B, Gloverin 2, and Lebocin 5 exhibit antifungal effects against *B. bassiana*, and Gloverin 2 and Cecropin A show synergistic antifungal activity. Most of these proteins also have antibacterial properties (Kaneko *et al*., 2008; Lu *et al*., 2016; Lu *et al*., 2017a; Lu *et al*., 2017b) [17, 26, 27, 28]. Serine protease inhibitors/serpins block melanization caused by CDEP-1, inhibit germination of *B. bassiana* conidia, and increase the survival of silkworm larvae infected with this fungus (Li *et al*., 2012; Li *et al*., 2015; Li *et al*., 2016) [22, 21, 20]. Thus, these proteins may play significant roles in defense against *B. bassiana* infection.

Expression of immune system against viral infection in silkworm

The digestive juice of silkworm larvae contains several antiviral proteins against BmNPV, including *B. mori* lipase Bmlipase-1 (Ponnuvel *et al*., 2003) [36], serine protease BmSP-2 (Nakazawa *et al*., 2004) [33], *B. mori* NADPH oxidoreductase BmNOX (Selot *et al*., 2007) [41], and red fluorescent proteins (Sunagar *et al*., 2011) [45]. Even if midgut epithelial cells are infected, the virus-containing cells can be removed by high-frequency apoptosis.

Intestinal microorganism play a role in the host defence system against viral pathogens, a lipase gene from the silkworm intestinal bacterium *B. pumilus* SW41 were characterized, and antiviral activity of its protein against *B. mori* nucleopolyhedrovirus (BmNPV) were tested. The total enzyme activity of this recombinant lipase reached 277.40 U/mg at the optimum temperature of 25 °C and optimum pH value of 8.0. The antiviral test showed that a relative high concentration of the recombinant lipase reduced BmNPV infectivity *in vitro*, which resulted in decreased viral DNA abundance and viral occlusion bodies (Liu *et al.*, 2018) [25].

Conclusion

This study provides information about microorganisms present in the silkworm gut. These beneficial microorganisms involved primarily for enzymes production in silkworm gut for digestion of ingested host plants. In addition to that, colonized microorganism produced many antimicrobial peptides, defensive proteins to protect the host from different pathogenic microorganisms in silkworm. However, those beneficial microorganisms applied in probiotic manner to silkworm which enhanced the all economic traits of silkworm and also increase the innate immunity of the silkworm. It suggested that, silkworm and their core intestinal microrganisms established a symbiotic relationship of co-adaptation and co-evolution to maintain survival and reproduction in the evolutionary process.

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