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INTERNATIONAL JOURNAL OF CURRENT RESEARCH

International Journal of Current Research
Vol.11, pp.192-199, December, 2010

ISSN: 0975-833X

RESEARCH ARTICLE

A STUDY ON THE ESTABLISHMENT OF BACTERIAL MICROBIOTA IN THE GUT OF SILKWORM *Bombyx mori.*

Sekar, P., Balasundaram, A* and George John.

Department of Zoology, Periyar E.V.R. College, Tiruchirappalli-620023, Tamil Nadu, India.

ARTICLE INFO

Article History:

Received 12th September, 2010
Received in revised form
15th October, 2010
Accepted 13th November, 2010
Published online 28th December, 2010

Key words:

Gut microflora;
Bombyx mori;
Silkworm.

ABSTRACT

A study was carried out to assess the colonization of bacterial strains adherent to the upper and lower sides of the mulberry leaves, in the intestinal zones of the silkworm *Bombyx mori.* Upper and lower sides of one week, two week and three week old mulberry leaves were scraped aseptically and cultured in nutrient agar medium. Morphological, physiological and fermentation characteristics of the bacterial isolates were studied to identify the strains. The bacterial strains isolated were: *Bacillus cereus, Bacillus subtilis, Lactococcus lactis, Staphylococcus lactis, Enterobacter aerogenes, Escherichia coli* and *Klebsiella pneumoniae.* Second instar larvae of *Bombyx mori* were segregated into three sets and each set was maintained on mulberry leaves of selected age(one, two or three week old) for 15 days and the regionwise abundance of gut bacteria was assessed. Among the intestinal zones of *Bombyx mori* foregut harboured more bacteria (5.2 to 6.2 cfu x 10^8). Identity and distribution of intestinal bacteria could establish that gut microbiota of *B. mori* has its origin from the phyllosphere microbes of mulberry and that the gut microbes may have definite role in the nutrition of silkworm.

INTRODUCTION

Indian silk industry is based largely on the mulberry silk worm, *Bombyx mori.* Economics of silk production depends greatly on the quality of cocoons produced by the worm (Krishnaswami and Sundaramullary, 1991), which inturn is dependant upon the nutritional demands of silk worm. As the demand for silk is ever increasing, it is imperative that sericulturists should find ways and means to improve the quality of silk, and the most plausible angle for this is the nutritional approach. Considering the phyllophagous nature of silk worm, depending solely on mulberry leaves, the options available are restricted to the improvement of nutritional quality of leaves, use of nutrient additives and supplementary ingredients which can be routed through mulberry leaves. Recent approaches in this direction include the application of VAM fungi and bacterial biofertilizers to improve the mulberry leaf quality and thereby the cocoon characters (Rao *et al.,* 2007) and the use of

***Corresponding author:** *balasundaram.va@gmail.com*

plant growth promoting rhizobacteria (Unni *et al.,* 2008). Supplementing mulberry leaf powder with starch, phosphate, vitamin B complex, Vitamin C, citric acid, sorbic acid etc., has met with limited success. Nutritional contributions and the symbiotic benefits offered by insect gut-dwelling bacteria (Dillon and Dillon, 2004; Yuan *et al.,* 2006) is yet another area which can substantially modify and promote the health and silk production capacity of *Bombyx mori,* although this field has a only attained limited attention in the sericulture scenario, notwithstanding the view of many scientists to consider associated microbiota as potential pathogens or contaminants. Bacteria are associated with a number of species across all major orders of Insecta (Buchner, 1965; Campbell, 1990; Dillon and Dillon, 2004) and the insect gut provides a suitable habitat for bacteria (Bignell, 1983).

There exists a mutualistic association between a number of insect species and their extracellular gut-microbiota, and they contribute to the nutrition of the host (Tanada and Kaya, 1993). An indigenous biota is present in all the individuals of a species and maintains stable climax communities in the intestinal mileu of the species. Many species derive the microbiota from the surrounding environment such as the phylloplane of food plants. Aphid gut microbes share a common ancestry with intracellular symbionts and bacteria ingested from food plant (Harada *et al.,* 1996). Vast microbial diversity exists among the gut micro-biota of termites (Okhuma and Kudo, 1996; Paster *et al.,* 1996; Kudo *et al.,* 1998). Presence of bacteria in the gut of mulberry silkworm (*Bombyx mori*) have been reported by Roy *et al.* (2000) and Kodama, (2001). Sittenfield *et al.* (2000) explored the gut bacteria and changes associated with dietary changes in the highly polyphagous tropical caterpillar *Antomeris zuguna*. Health promoting and immuno-stimulating effects of commensalic microbes has been established in mammals and in fisheries. These bacteria are termed 'probiotic' meaning 'prolife': a word derived from Greek language (Schrezenmeir and de Vrese, 2001). A widely accepted definition for probiotic by Fuller (1989) goes as following "Probiotics are live microbial feed supplement which beneficially affects the host animal by improving its intestinal microbial balance". Important reviews of Fuller (1989; 1992), Gilliland (1990), Gibson (1994), Tannock (1995) and Ouewehand *et al.* (2002) reveal the benefits of probiotics and their diverse actions on farm animals and man. Reports on the probiotic potential of *Lactobacilllus plantarum* (Singh *et al.,* 2005), and endogenous actinomycete, *Streptomyces noursei* (Subramanian *et al.,* 2009) are the two available works in the Indian sericulture scenario.

Although sericulture has been practiced from time immemorial, a microbiotic inventory of their gut or the study on the sources intestinal bacteria has never been a priority. This study is a basic step in the intestinal microbiotic characterization of the Indian silkworm, *Bombyx mori* and to assess its relation with the hosts's feeding habit and environment.

MATERIAL AND METHODS

Mulberry leaves selected for the present study belong to Mysore local variety M5. Their foliage growth pattern was studied, so as to enable the segregation of one week, two week and three week-old leaves. Fresh leaves of each age were collected daily and fed to the silk worms, *ad libitum* (Krishnaswami *et al.,* 1978). *Bombyx mori* (Lepidoptera) LxNB4D2, a crossbreed of a local and multivoltine variety was used for the experiments. Second instar larvae were obtained from the Tamilnadu State Government Silk Rearing Centre, Manikandam, Tiruchirappalli. The larvae were maintained in bamboo trays lined with newspaper sheets and covered by wiremesh-lids. The trays were kept on moistened jute bags to maintain cool, humid conditions (27±2°C, relative humidity: 70±5%). Care was taken to keep away natural enemies like rats and lizards. Three duplicate sets of 10 larvae of second instar stage were maintained for the experiment. First set was fed with one week-old mulberry leaves, the second with two week old leaves and the third with three week-old leaves. Feeding was done on a daily basis through out the experiment period of 15 days. After fifteen days, guts of the larvae were aseptically isolated mashed in sterile physiological saline and the bateria were enumerated by pour

plate technique in Nutrient agar (Himedia, India) as the culture medium.

Bacterial isolation

Mulberry leaves: Freshly plucked mulberry leaves of varied age groups (I, II, III weeks old) were used for bacterial analysis. One cm^2 area from the upper and lower sides of the leaves was scraped by a sterile knife and the scrapings were mixed thoroughly in one ml physiological saline. These saline – leaf scraping mixtures were serially diluted in 0.9% saline for the culture of leaf dwelling heterotrophic bacteria in Nutrient Agar culture medium.

Larvae: After 15 days of feeding with appropriately aged mulberry leaves, the three groups of larvae were subjected to bacterial analysis. The larvae were dissected with sterile instruments in laminar airflow chamber and the entire gut was separated. The gut was separated into three portions namely the foregut, mid gut and hind gut. Each part was approximately 1cm in length. The separated portions were put in sterile 5ml beakers and 1 ml sterile saline was added to each. The gut portions were macerated in the saline with a sterile glass rod, so as to disperse the gut bacterial flora into the saline tissue mixture. These saline – tissue mixtures were serially diluted with sterile saline for culturing and enumerating bacteria.

Bacterial culture

Nutrient agar media and nutrient broth were used to culture the bacteria from mulberry leaves and silkworm gut. . The medium was poured into petri dishes and the serially diluted samples were inoculated aseptically into the medium at room temperature. The pertiplates were incubated at room temperature (29±1°C) for 36 hours and the developed bacterial colonies were studied for their morphology. They were enumerated under illumination with a colony counter. Colonies were segregated based on their morphology and were streaked on agar slants. Agar slants were incubated at room temperature (29±1°C) for 36 hours and then stored at 5°C. These pure culture colonies were used for identification using substrate utilization-screening and carbohydrate fermentation tests outlined by MacFaddin (1980) and in Bergey's manual of Determinative Bacteriology (Holt *et al.,* 1994).

Statistical analysis

Distribution of heterotrophic bacteria based on the age of mulberry leaves and the zonewise distribution in the *Bombyx mori* larval gut were subjected to two way Analysis of Variance and Student Newman Keul's test (SNK-test), using a statistical package-(SPSS version–10).

RESULTS

Bacterial flora of mulberry leaves: Total heterotrophic bacteria of 1 – 3 weeks old leaves of mulberry varied from 0.7 to 3.4 cfu x 10^8 sq. cm. Young (one week old) leaves harboured greater bacterial population while the older ones showed a decreasing trend in the numerical abundance of bacteria (Table – 1). Statistical analysis also supported the above observations. Compared to the upper side of the leaves, the lower side had better representation of bacterial flora.

B*acteria-Species composition*: Dominant bacterial species and their numerical abundance on mulberry leaves are presented in Table 2. In all the mulberry leaves of different age groups, *Bacillus cereus* formed the dominant species, although their numerical status decreased with increase in age of the leaf. In one-week old leaves, *Bacillus cereus*, *Enterobacter*, *Lactococcus lactis* and *Staphylococcus lactis* were the dominant strains, of which *B. cereus* (4.2cfu x 10^7) exhibited maximum abundance. Two week old leaves also showed a similar trend, although the number of bacteria was lesser. Besides, the dominant *B. cereus*, the other strains represented largely were *Lactococcus lactis*, *Enterobacter aerogenes* and *Escherichia coli* (Table 2). Three week – old mulberry leaves were low in bacterial number and diversity. Along with *B. cereus*, other species such as *Klebsiella pneumoniae* and *Escherichia coli* were also distributed, although in comparatively meagre number. Upper and lower sides of leaves did not show any variation with regard to species composition

Table 1. Viable microbial population on Mulberry leaves (values in cfu x 10^8 per cm^2)

Leaf-age	Upper side	Lower side
One week	2.8^a	3.4^a
Two weeks	1.6^b	1.8^b
Three weeks	0.7^c	0.8^c

F= 250.29 (P< 0.001), Dissimilar superscripts denote, significantly different values statistically.

Table 2. Dominant bacterial species on Mulberry leaves (upper and lower sides)

Leaf-age	Bacterial species	Number cfu x10^7
One week	Bacillus cereus	4.2
	Enterobacter	2.2
	Lactococcus lactis	1.8
	Staphylococcus lactis	1.7
Two weeks	*Bacillus cereus*	2.8
	Lactococcus lactis	1.4
	Enterobacter aerogenes	0.8
	Escherichia coli	0.4
Three weeks	*Bacillus cereus*	2.0
	Klebsilella pneumoniae	0.2
	Escherichia coli	0.4

Staphylococcus lactis, *Escherichia coli*, *Klebsiella pneumoniae*.

Bacterial Colonization in the gut of *Bombyx mori* larvae

Region-wise abundance: Region – wise abundance of total heterotrophic bacteria in the gut of *Bombyx mori* is presented in Table 5. Among the gut regions, foregut, midgut and hind gut, the foregut zone was found to be inhabited by greater number of bacteria (5.2 to 6.2 cfu x 10^8). Bacteria were at low number in the lumen of midgut and hind gut (3.8 to 4.9 cfu x 10^8). Region wise bacterial colonisation in *Bombyx mori* – larval intestine was significantly different statistically (P<0.01), and the foregut was distinctly ahead in microbiota abundance than the other two intestinal zones. Age of the mulberry leaves had an apparent retrogressive influence on intestinal colonization, however this decrease was not significant statistically.

Intestinal dominance of bacterial strains

Among the bacterial strains, *Bacillus cereus* was found to colonise all the zones of the gut;

Table 3. Characteristics of bacterial strains

S. No	*Organism*	*Gram stain*	*Morphology*	*Endospores*	*Capsular formation*	*Motility*	*Colony morphology*	*Optimum temperature °C*	Oxygen requirement
1.	*Lactococcus lactis*	+	Coccus: pairs, short chains	-	-	-	Small, pinpoint size, translucent to white	30	Fac. An
2.	*Bacillus cereus*	+	Bacillus: singles,pairs, chains	+	+	+	Spreading, smooth, white	37	Aerobe
3.	*Bacillus subtilis*	+	Bacillus: singles, pairs	+	+	+	Spreading, white	37	Aerobe
4.	*Escherichia coli*	+	Bacillus: short, singles	-	-	+	Smooth, white to cream	37	Fac An
5.	*Enterobater aerogenes*	+	Bacillus: short, singles	-	-	+	Smooth, mucoid, white	37	Fac An

Fac. An – Facultative anaerobe

Bacterial characteristics

Characteristics of the bacterial strains were revealed through staining and microscopic observation and by various biochemical tests (Table 3 and 4) and the characterized bacterial strains were *Bacillus cereus*, *Bacillus subtilis*, *Enterobacter aerogenes*, *Lactococcus lactis*, particularly their dominance was discernible at the midgut region. *Bacillus subtilis* ranked second in the colonization of the gut regions, while the other strains like *Lactococcus lactis*, *Klebsiella pneumonia*, *Enterobacter aerogenes* and *Escherichia coli* were discontinuously distributed among the gut regions (Table 6).

Table 4. Fermentation properties of bacterial strains isolated from mulberry leaves.

S.No	*Organism Name*	*Hemolysis*	*Catalase*	*Glucose*	*Lactose*	*Sucrose*	*Mannitol*	*Sorbitol*	*Inole*	*Citrate*	*Gel*	*MR*	*V-P V-p*	*TSI-butt*	*TSI-slant*
1	*Lactococcus lactis*	α/γ	-	A	A	<A>	-	-	-	-	-	ND	ND	A	A
2.	*Bacillus cereus*	β	+	A	-	A	-	-	-	+	+	ND	+	+/-A	-
3.	*Escherichia coli*	γ	+	A/G	A/G	Var	A/G	A/G	+	-	-	+	-	A/G	A
4.	*Enterobacter aerogenes*	γ	+	A/G	A/G	A/G	A/G	A/G	-	+	-	-	+	A/G	A
5.	*Klebsiella Pneumoniae*	γ	+	A/G	A	A	A	A-	-	+	-	-	+	A/G	A
6.	*Bacillus subtilis*	α/γ	+	A	A	-	A	-	-	+	+	ND	+	A	A

+Positive – Negative A = Acid production AG = Acid and Gas productionVar- variable; MR- Methyl red test; VP- Voges-Proskauer; Gel- Gelatin liqufication;

Table 5. Total heterotrophic bateria in the intestinal zones of *Bombyx mori* larvae fed on Mulberry leaves (Number as cfu x10^8 per 1cm^2 zone)

Leaf age	Intestinal zones		
	Fore gut	Mid gut	Hind gut
One week	6.2^a	4.7^b	4.0^b
Two weeks	5.6^a	4.5^b	3.8^b
Three weeks	5.2^a	4.9^b	4.1^b

F = 25.46 (P < 0.01). Dissimilar superscripts denote significantly different values (SNK test).

Table 6. Dominant bacterial species in intestinal zones of *Bombyx mori* larvae fed on Mulberry leaves

Bacterial species	One week			Two weeks			**Three weeks**		
	Intestinal zones								
	FG	**MG**	**HG**	**FG**	**MG**	**HG**	**FG**	**MG**	**HG**
Bacillus cereus	+	++	++	+	++	+	+	++	+
Lactococcus lactis	+	-	-	+	+	-	-	+	-
Klebsiella pneumoniae	-	-	-	-	-	-	+	-	-
Enterobacter aerogenes	-	+	+	-	+	+	-	-	-
Escherichia coli	-	-	-	-	-	-	+	-	-
Bacillus subtiius	-	+	+	-	+	-	-	+	-

+ = Present; + + = Dominant; - = Absent; FG – Fore gut; MG – Mid gut; HG – Hind gut

DISCUSSION

Leaf –microbe ecosystems are poorly understood, but it is estimated that each leaf might be the habitat for at least one to ten million bacteria and a number of factors affect the ecology of the phyllosphere microbiology at a given time (Hirano and Upper, 2000). These factors exist in a continuously changing environment and include among others, plant and bacterial genetics, leaf surface and topography, plant chemistry, weather and plant phenology (Hirano and Upper,2000).

Plants and insects co-existed for atleast 100 million years, producing a great diversity of beneficial and negative relationship (Stotz *et al.,* 1999, Dillon *et al.,* 2000). Gut microbiota is regarded as a valuable metabolic resource for insects on suboptimal diets, but apart from this, most relationship between insects and their microbiota remain undefined. Microbial transformation of plant secondary compounds in an insect gut and adaptation by the host to use the resulting common metabolites are unique to insects (Dillon, 2000). The present inventory probe on the mulberry leaf microbiota has revealed that, their source is the soil in which the plants are cultivated, and the farmyard manure used as fertilizers. Strains of the *Bacillus* genus commonly occur in soil, while the other representatives like *Enterobacter*, *Lactococcus*, *E. coli*, are all reported frequently in the colon of vertebrates. Influence of faecal microbes and bacteria from decomposing debris of biological materials on the leaf-microbe ecosystem has been discussed by Sittenfeld *et al.* (2002).

Almost all animals possess their own gut microflora consisting of a number of bacteria and other microbial species in their alimentary tracts. Most of the gut bacteria are parasitic or commensal associates of the host organisms, but some of them have beneficial effects on the hosts (Xu and Gordon, 2003; Dillon and Dillon, 2004; Ley *et al.,* 2006). Insects seems to acquire specific bacterial symbionts of a beneficial nature from the environment. In the present study, the colonized bacteria in the intestine of silkworm were a reflection of the mulberry leaf ecosystem, although their colonizing capacity varied according to the region of silkworm gut. Strains of *Bacillus*, the dominant gut colonizer in the present study, are reported to have probiotic properties when they became residents of the gut of marine shrimps (Rengipat *et al.,* 1998). Similar beneficial effects of lactic acid bacteria are reported by Ouwehand *et al.* (2002). Hence it is probable that at least a few of the gut dwelling microbes have beneficial effect on silkworm. In general, most of the bacterial genera and species reported in the present study have been frequently isolated from animal intestines, including insects like termites, mosquitoes, flies and crickets (Eutick *et al.,* 1978; Demaio *et al.,* 1996; Kadavy *et al.,* 1999; Ulrich *et al.,* 1981). Among the 22 species isolated from gut microbiota of polyphagous tropical caterpillar *Antomeris zugana*, *Enterococcus* represents 81%, followed by *Bacillus* species (Sittenfield *et al.,* 2002). Similarly in the present study also *Enterobacter* were observed in 1-2 week old leaves and also in the three gut regions of silkworm. *Enterobacter cloacae* has been reported to survive and grow in the guts of silkworms (Watanabe *et al.,* 2000). Broderick *et al.* (2009) reported that *Lactococcus lactis* and *Enterobacter* are present widely in the gut microbiota of lepidopteran insects; the same two species were isolated in the present study also.

The other predominant bacterial species such as *Klebsiella*, *Staphylococcus*, *Bacillus* species, reported in the present study, could also be found in the gut microbiota of variety of insects (Zhou *et al.,* 2004; Yu *et al.,* 2008). Enterobacteriacea and *Staphylococcus* have been reported in the intestine of the termite and silkworm (Moriya and Toshiaki, 1996; Yuan, 2006). Demio *et al.* (1996) isolated *Bacillus*, *Enterococcus* and *Klebsiella* in the gut microbiota of *Automeris zugana*. Microbes have contributed a great deal towards success of insects. Gut is always in dynamic transition such that, the intestinal cells move up the microvilli and as they reach the peak, they are sloughed off. Even then insects used to establish its own microbiota mostly related to its food. The result of this present study, although basic, indicates the role of insect gut microbial communities in the interaction between the silkworm, the microbial phyllosphere and the age of the mulberry leaves.

Acknowledgements

Mr. P. Sekar, acknowledges the financial support extended by the University Grants Commission, through Rajiv Gandhi National Fund Scholarship.

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