Int. J. Indust. Entomol. Vol. 4, No. 2, 2002, pp. 127~131

International Journal of *Industrial Entomology*

Midgut Microflora of Pure Mysore (Multivoltine) and NB_4D_2 (Bivoltine) Silkworm (*Bombyx mori* L.) Races During Late Larval Instars

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(Received 11 March 2002; Accepted 23 April 2002)

The qualitative and quantitative changes of bacterial flora associated with the Pure Mysore (Multivoltine) and NB_4D_2 (Bivoltine) silkworm (*Bombyx mori* L.) midgut during third, fourth and fifth instars were studied. Larvae reared on mulberry leaves were dissected and their midgut bacterial populations were enumerated through serial dilution technique and after 72 hrs of incubation period at 28 ± 1°C, the bacterial population was estimated. The results showed a highest mean value of 15×10^6 CFU/g and 28×10^6 CFU/g in Pure Mysore and NB_4D_2 races, respectively, in midgut tissue of fifth instar larvae. The natural epiphytic microflora of mulberry leaves fed during the respective instars was also studied and found maximum 14×10^3 CFU/g in leaves fed in third instars, followed by 5.3×10^3 CFU/g and 2.1×10^3 CFU/g in leaves fed during fourth and fifth instars, respectively. The bacterial flora colonized in midgut was found to be elaborating amylase, caseinase, gelatinase, lipase and urease enzymes. The highest percentages of isolates were amylase producers followed by protein and lipid splitters in Pure Mysore, whereas in NB_4D_2 protein splitter were dominated followed by lipase and amylase producers in NB_4D_2. The results indicate that the natural microflora may play a vital role in the digestion of ingested food materials in silkworms.

Key words : *Bombyx mori*, Multivoltine, Bivoltine, Mulberry, Midgut, Microflora.

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Introduction

Bivoltine silkworms suitable for temperate climatic zones, are known to produce both quantitative and qualitative silk. In spite of its high productivity, bivoltines are having poor adaptability to the fluctuation temperature conditions and other stress conditions and are also susceptible to viral and bacterial diseases, which are the major cause for failure of the crop at the farmers level (Samson *et al.*, 1990; Nataraju *et al.*, 1998). The multivoltine races have a unique character to adjust to the adverse climatic conditions and are tolerant to different types of diseases in comparison to bivoltines.

Major diseases of silkworm are Nuclear Polyhedrosis Virus (NPV), flacheriae, pebrine and muscardine. Bacterial disease causes a significant loss to the population and it has been reported that there is synergistic effect of bacteria in association with a Picarnovirus namely, *Bombyx mori* infectious flacherie virus (BmIFV) up to 48 - 56% on mortality due to IFV (Nataraju *et al.*, 1999).

Leaf surfaces of various plants including mulberry (*Morus* spp) harbors number of epiphytic bacteria (Lindow *et al.*, 1978; Lindow and Andersen, 1996; Takahashi *et al.*, 1995). Reports are also available on the presence and colonization of epiphytic bacteria in the guts of several arthropod insects including silkworm (Brooks, 1963; Iwanami and Ono, 1963; Watanabe *et al.*, 1996). These facts suggest that some natural micro flora may colonize in the gut of insects as symbionts or competitors. The possibility to have a possible role in the production of diseases is not clearly understood. However, the literature on the role of heterotrophic bacteria associated with stages of the silkworm larvae are very scanty. Hence the present study was undertaken to find out the midgut bacterial flora of Pure Mysore (Multivoltine) and NB_4D_2 (Bivoltine) silkworms, *Bombyx mori*, during its third, fourth and fifth instars, and the natural microflora of mulberry fed during the respective instars were also studied.

Materials and Methods

Sample collection

Pure Mysore (Multivoltine) and NB_4D_2 (Bivoltine) races of *B. mori* silkworm were reared in silkworm rearing house as per the recommended silkworm rearing practices (Krishnaswamy, 1978). Five larvae of each race in different instars were collected in sterile polythene containers. The larvae were surface sterilized with 70% alcohol and dissected under aseptic condition for the collection of midgut tissue. The mulberry leaves fed to the silkworm during different instars were also collected in sterile polythene bags and simultaneously analyzed for heterotrophic bacteria.

Sample preparation

The collected midguts ware weighed and homogenzed in the sterile glass homogenizer with distilled water. Homogenized samples were serially diluted in sterile double distilled water and plated on nutrient agar media. Five individual midguts in three replications of each instar were used for sample preparation. To analyze the microflora of the mulberry leaves, one g of leaf was collected directly from the plant and weighed under aseptic conditions and homogenized in a presterile homoginizer in sterile double distilled water. Homogenized samples of midguts and mulberry leaves were serially diluted using sterile double distilled water and plated on nutrient agar media in triplicate.

All the inoculated plates were incubated at 28 ± 1°C for 72 hrs, after which the bacterial population was determined by counting the colony forming units (CFUs). The bacterial load was expressed as CFU per g. wet weight of the midgut or leaf. Colonies were sampled at random from each of the agar plates and purified by streaking on nutrient agar media.

Characterization of isolates

Isolated pure cultures of bacteria were tested for the following morphological and biochemical properties: shape, gram stain, spore stain, motility, kovacs oxidase test and oxidation fermentation and identified according to Buchanan and Gibbons (1979). To identify the digestive enzymes produced by the isolates, their ability to utilize amylase, gelatinase, lipase, caseinase and urease was determined by plate assay (Cowan and Steel, 1970; Edwards and Ewing, 1972).

Results

The mean heterotrophic bacterial population observed in the midguts of Pure Mysore and NB_4D_2 silkworm races are shown in Table 1. The result shows that a higher heterotrophic bacterial population in the midguts of the later instars (third, fourth and fifth) in both races. The highest mean value of 15×10^6 CFU/g was observed in the midguts of Pure Mysore and 28×10^6 CFU/g in case of NB_4D_2. The mean heterotrophic bacterial population of mulberry leaves fed to the various instars showed a little variation. Leaves fed during fourth instar showed highest mean value of 14×10^3 CFU/g, whereas leaves fed in third instar showed an average mean value of 5.3×10^3 CFU/g and least mean value of population 2.1×10^3 CFU/g was noticed in leaves fed during fifth instar.

Table 1. Heterotrophic bacterial population in the midguts of Pure Mysore and NB_4D_2 races and mulberry leaves fed to silkworms

Source	Mean heterotrophic bacterial population	
	Pure Mysore	NB_4D_2
G1	$63 \times 10^4 \pm 3.44$	$94 \times 10^4 \pm 3.81$
G2	$27 \times 10^5 \pm 2.79$	$41 \times 10^5 \pm 3.09$
G3	$15 \times 10^6 \pm 2.40$	$28 \times 10^6 \pm 2.81$
M1	$5.3 \times 10^3 \pm 1.85$	$53 \times 10^3 \pm 1.85$
M2	$14 \times 0^3 \pm 2.36$	$14 \times 10^3 \pm 2.36$
M3	$2.1 \times 10^3 \pm 1.80$	$2.1 \times 10^3 \pm 1.80$

G1, G2, and G3 represent the midguts of third, fourth and fifth instar larvae, respectively.
M1, M2 and M3 represent the mulberry leaves fed to the third, fourth and fifth instar larvae, respectively.

The quantitative nature of the midgut microflora of Pure Mysore and NB_4D_2 during third, fourth and fifth instars along with natural microflora of mulberry leaves fed during the respective instars was shown in the Figure 1. It was observed that the midgut microflora of different instars in both the races was dominated by *Bacillus* followed by *Micrococus* and *Corynebacterium* except in case of Pure Mysore mid gut tissue of fourth and fifth instars. *Micrococcus* dominated followed by *Corynebacterium* and *Bacillus* in fourth instar midguts of Pure Mysore and in fifth instar *Bacillus* followed by *Corynebacterium* and *Micrococcus*. Other genera encountered were *Enterobacteriaceae, Moraxella, Alcaligenes, Acinetobacter* and *Aeromonas*. On mulberry leaves, *Micrococcus* was dominated genus followed by *Corynebacterium* and *Bacillus*. Other genera such as *Moraxella, Alcaligenus, Acinetobacter, Aeromonas* and members of the family *Enterobactriaceae* were also recorded.

The morphological and physiological characteristics along with the capacity of elaboration of the enzymes by

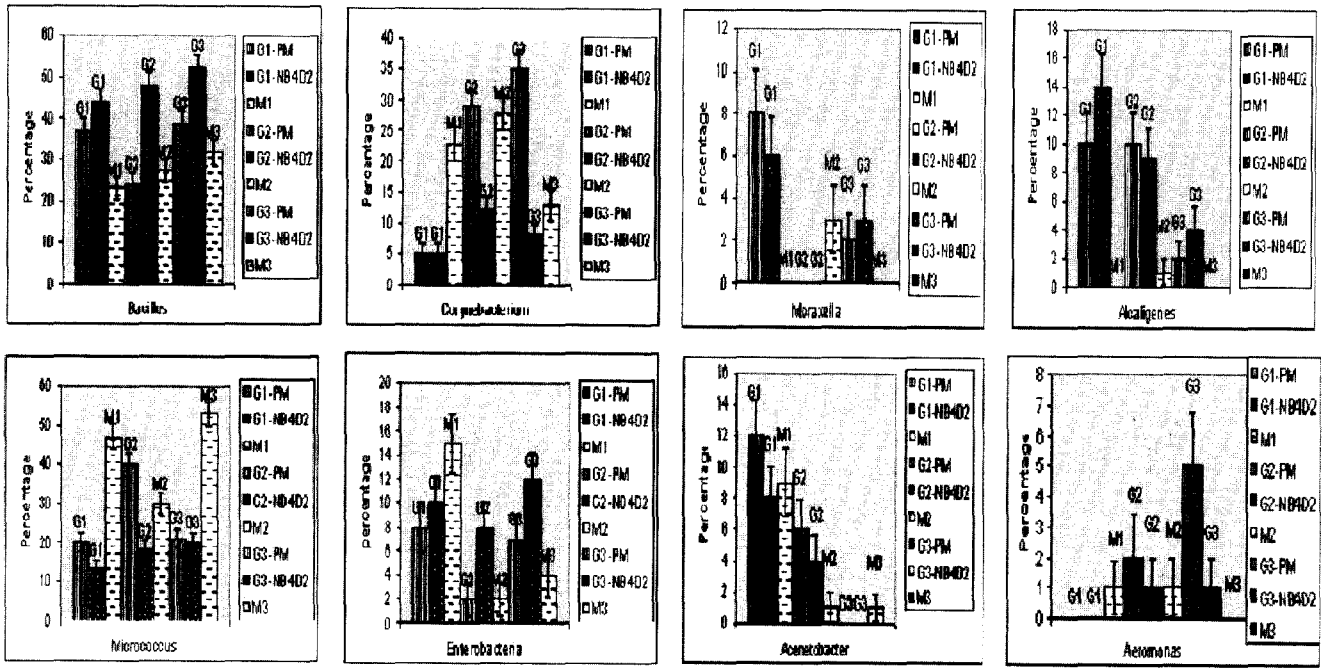

Fig. 1. Bacterial genera percentage occurred in the midguts of Pure Mysore (PM) and NB_4D_2 silkworm races on mulberry leaves fed to silkworm in respective instars. G1, G2 and G3 represent the midguts of third, fourth and fifth instar larvae, respectively. M1, M2 and M3 represent the mulberry leaves fed to the third, fourth and fifth instar larvae.

Table 2. Percentage of bacterial isolates from the Pure Mysore (PM) and NB_4D_2 silkworm midguts and mulberry leaves showing a positive response to the characterization tests and producing the assayed digestive enzymes

	G1		G2		G3		M1	M2	M3
	PM	NB_4D_2	PM	NB_4D_2	PM	NB_4D_2			
Characteristics									
Rods	68	80	73	83	65	70	59	67	70
Cocci	32	20	27	17	35	30	41	33	30
Gram positive rods	70	75	69	74	89	90	63	90	78
Gram nagative rods	30	25	31	26	11	10	37	10	22
Motility	28	32	38	45	30	36	34	26	-
Kovacs oxidase	32	41	35	43	67	76	9	51	42
Non fermentative	64	58	63	68	53	58	72	70	100
Fermentation of Glucose	30	45	34	48	36	42	19	25	-
Alkaline	8	9	4	14	-	8	6	9	-
Digestive Enzymes									
Amylase	55	13	53	11	58	9	22	20	34
Caseinase	48	58	54	48	49	45	41	68	43
Gelatinase	39	44	28	39	31	41	19	21	51
Lipase	44	45	25	42	26	44	38	62	31
Urease	49	56	19	44	9	32	36	63	30

G1, G2 and G3 represent the midguts of third, fourth and fifth instar silkworms, respectively.
M1, M2 and M3 represent the mulberry leaves fed to the third, fourth and fifth instar silkworms, respectively.

the isolates of bacterial cultures are shown in Table 2. The results showed a predominance of rod shaped forms over Cocci and Gram positive forms over the Gram negative bacteria in the midguts of both races during all instars. A

considerable percentage of the isolates were motile and were able to ferment glucose. Highest number of Pure Mysore midgut bacterial isolates produced amylase whereas caseinase producers were observed highest in NB_4D_2 midgut isolates.

Isolates of mulberry leaves also showed highest number of rods, Gram positive than the Cocci and Gram nagative organisms. Maximum isolates from the mulberry leaves fed during third and fourth instar larvae were motile and were able to ferment glucose. Caseinase producers observed in leaves fed during third and fourth instar, whereas gelatinase producers followed by caseinase producers were found in leaves fed in fifth instar.

Discussion

The results revealed that the bacterial flora of the silkworm midguts showed an increase in the heterotrophic bacterial population with the increase of age of silkworm in both the races. The highest percentage of colonization *i.e.*, 29.78% was observed in NB_4D_2 whereas 23.80% observed in Pure Mysore during fifth instar when compare to third instar midgut microbial population. This may be due to higher feeding activity of NB_4D_2 over PM.

Chitra *et al.* (1975) noticed a higher frequency of bacterial flacherie in fifth instar silkworm. They attributed this to a higher food intake, which resulted in entry of more microbes. The results indicate a random occurrence of various genera, except for *Bacillus, Micrococcus* and *Corynebacterium* in the midguts of various larval instars of the both the races. These genera of microflora were also observed on mulberry leaves fed to silkworm. This suggests that the bacteria enter through food and then they are able to colonize in the midgut. This contradicts the observations of Harris *et al.* (1991) who reported a distinctive gut microflora in some arthropods. However, Plante *et al.* (1989) fed vibrio and Pseudomonas bacteria to a polycheate and observed rapid growth and colonization of the ingested bacteria in the hindgut of the animal. The gut of the silkworm, which feeds only on mulberry leaves, is a suitable habitat for *E. herbicola* of the most common epiphytic bacteria on mulberry leaves. (Takahashi *et al.*, 1995). Bacillus group of bacteria, which was predominant up to 52 percent was observed in the fifth instar midguts of NB_4D_2. This may be the cause for high susceptibility of NB_4D_2 race to diseases compared to Pure Mysore. Nataraju *et al.* (1999) reported the association of *Bacillus* spp in combination with BmIFV caused mortality ranging to an extent of 48 to 56 percentage.

The results of the present study showed that many of the isolates recovered from the midgut of the various instars and from the leaf fed were capable of elaborating different enzymes like amylase, caseinase, gelatinase, lipases and ureases. Amylase producers were predominant in Pure Mysore whereas caseinase producers in NB_4D_2 were observed.

The gut of arthropods is generally suitable for colonization by certain microbes (Bignell, 1984). Symbiotic microbes have also been reported in the gut of insects and arthropods (Brooks, 1975; Harris *et al.*, 1991) ingested bacteria and the enzymes they produce can contribute significantly to the digestion of various food types (Cruden and Markovetz, 1979; OBrien and Slaytor, 1982). The enzymes elaborated by the bacteria present in the silkworm gut may assist in digestion and assimilation of food. Hence, there is a need to take up further studies to isolate specific bacterial isolates for better food conversion efficiency and to use as synergistic or antagonists for management of silkworm diseases with special reference to bivoltine silkworms to boost the bivoltine silk production in the silk producing countries.

Acknowledgments

The authors express their thanks to Dr. K. Ramaswamy, Scientist-C, CFTRI, Mysore in helping the identification of the bacterial isolates and Sri. Arnab Bhattacharjee, CSR & TI, Mysore for helping in the preparation of graphs.

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