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M Chandana
Department of Sericulture, College of Agriculture, UAS, GKVK, Bengaluru, Karnataka, India

RN Bhaskar
Department of Sericulture, College of Agriculture, UAS, GKVK, Bengaluru, Karnataka, India

Fatima Sadatulla
Department of Sericulture, College of Agriculture, UAS, GKVK, Bengaluru, Karnataka, India

Corresponding Author:
M Chandana
Department of Sericulture, College of Agriculture, UAS, GKVK, Bengaluru, Karnataka, India

Effect of Per-oral inoculation of *Bm*CPV and flacherie bacterial isolates on rearing parameters of silkworm, *Bombyx mori* L. (PM×CSR$_2$)

M Chandana, RN Bhaskar, and Fatima Sadatulla

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Abstract
Flacherie is considered to be a major disease in silkworm and it is a condition in which worm becomes flaccid and suffer from vomiting and dysentery. The disease is common, during summer and rainy seasons in all the sericultural areas of Karnataka and it is caused by different species of bacteria, viruses and their mixed infections. Per oral inoculation of different pathogenic bacteria *viz*., *Acinetobacter baumannii* (A), *Bacillus pumilus* (Bp), *Bacillus licheniformis* (Bl) and CPV (C) revealed significant in per cent reduction in larval weight over sterile water control, larval duration (days), moulting duration (hours) and per cent worms entering to subsequent instar. The maximum per cent larval weight reduction (43.50%), total larval duration (21.55 days), total moulting duration (107.12 h) and least per cent worms entering to subsequent instar (42.67%) was recorded in four organism treatment combination (T_7). Whereas, the minimum per cent larval weight reduction (19.62%), total larval duration (18.88 days), total moulting duration (80.94 h) and highest per cent worms entering to subsequent instar (90.00%) was noticed in T_1 in third instar inoculated batch. While the same trend was recorded in fourth and fifth instar inoculated batches. Irrespective of the group of bacterial isolate administered, more reduction in all the rearing parameters was observed in the silkworm batch inoculated with four organisms (T_7), followed by triple inoculation (T4,5,6and less reduction was recorded in dual inoculated (t1,2,3) batches.

Keywords: Silkworm, Per-oral inoculation, *Bm*CPV, Flacherie, Rearing parameters

Introduction
Silk is naturally produced animal fibre, which is known for its luster and grandeur. No other fabric can match it in luster and elegance, till today. In India, silk industry has made a significant progress in global silk production with an annual production of 33,840 MT (2018-19) of raw silk. India secured second position in global silk production with China being the leader in raw silk production (Shelagh, 2004) [10].

Silkworm are affected by many diseases which are owed to various biological, chemical, physical, nutritional and environmental causes. Silkworms being poikilothermic organisms, respond very quickly to the vagaries in environment, particularly to temperature and relative humidity. One of the major constraints in silk production are the diseases in silkworm rearing. About 30 - 40 per cent of cocoon crop was lost due to diseases in India (Vaidya, 1960) [13]. The most common among them are grasserie, flacherie (both bacterial and viral), muscardine and pebrine (Dasgupta, 1950) [5].

Flacherie has been considered to be the most serious malady of silkworm in India. The term flacherie refers to the flaccid condition of silkworm larvae suffering from dysentery. Flacherie may be due to viruses namely, nuclear polyhedrosis virus, cytoplasmic polyhedrosis virus, infectious flacherie virus and densonucleosis viruses (Aruga, 1971; Yokoyama, 1963) [3, 14] and different bacteria (Chitra *et al*., 1975) [4] and also mixed infection of virus and bacteria. The primary infection is due to the pathogen that alone can modifies the physiology as well as the whole system of the host. Whereas, secondary infection occurs during or after treatment for another infection. Secondary infections are usually bacterial infections which occurs in a host which is already been infected, usually with a virus.

Flacherie is also caused due to mixed infection of bacteria and virus (BmCPV). Amit Srivastava and Venkatesh Kumar (2009) [1] reported that 48.9 per cent and 35.4 per cent crop loss was recorded in the commercial silkworm rearing was due to the incidence of bacterial

Flacherie and cytoplasmic polyhedrosis, respectively. Flacherie was reported to cause massive cocoon crop loss of about 27-35 per cent with decrease in yield of cocoons 11 to 15 kg/100dfls (Selvakumar and Savithri *et al*., 2012) [8]. There are few reports on the possible synergistic association of bacteria and viruses in causing flacherie under laboratory conditions (Sivaprasad *et al*., 2000; Selvakumar *et al*., 2009) [11, 9]. Hence the present study was conducted on the effect of per-oral inoculation of BmCPV and flacherie bacterial isolates on rearing parameters of silkworm (Bombyx mori L.)

Material and Methods

The effect of per-oral inoculation of bacterial isolates and virus on rearing parameters of silkworm (PM × CSR2) was carried out at the Department of Sericulture, UAS, GKVK, Bengaluru during 2018-19. Three bacterial isolates which were isolated from the midgut BmCPV infected silkworm, molecular characterized and identified using 16S rRNA gene technology (viz., Acinetobacter baumannii, Bacillus pumilus and Bacillus licheniformis) were used in the present study.

Treatment details

T1- CPV + Acinetobacter baumannii
T2- CPV + Bacillus pumilus
T3- CPV + Bacillus licheniformis
T4- CPV + A. baumannii + B. pumilus
T5- CPV + B. pumilus + B. licheniformis
T6- CPV + B. licheniformis + A. baumannii
T7- CPV + A. baumannii + B. pumilus + B. licheniformis
T8- Normal leaf (Untreated control)
T9- Sterilized leaf (Control)

Enumeration of polyhedral bodies (no. /ml) and bacterial colony (CFU/ ml)

The viral suspension was collected and polyhedral bodies per ml was enumerated using Neubauer's haemocytometer. Before inoculation, the number of colony forming unit per ml was counted by digital colony counter from a pure culture colony of twenty four hours of incubation period. From these pure culture plates the stock suspension was prepared and their OD value was 1 at 600nm absorbance, under ELISA using SKANLT software version 4.1. The number of bacterial isolates in each purified plate at 10-7 dilution were enumerated by plating 10 micro liter of 10-7 serial diluted culture on nutrient agar medium using spread plate method. After 24 hours of incubation the number of colonies were counted using HIMEDIA digital counter pen and CFU/ml was calculated based on standard formula.

$$\text{CFU/ml} = \frac{\text{No. of colonies} \times \text{dilution factor}}{\text{Volume of culture for plating}}$$

Treatment Schedule

The known volume of inoculum was administered to silkworm at the rate of 0.5 ml per 50 larvae in each replication at the beginning of each instar of third, fourth, fifth and middle of the each instar. The inoculum was administered as follows:

In the present study, first organism is CPV in all the treatments, which was administered immediately after every moult, in third, fourth and fifth instar inoculated batch. While second organism was administered at the middle of respective instars and third organism was inoculated six hours after inoculating second organism in case of third and fourth instar. Whereas in fifth instar inoculated batch second organism was inoculated 48 hours after first organism (in case of two organism treatments) and third organism was inoculated 6 hours after second (in case of three organism treatment). In case of four organism combination (viz., virus + three bacteria) first organism at the beginning of instar, second organism after six hours, third organism at the middle of the instar and fourth was administered, six hours after third organism administration in third instar inoculated batch. Further in fourth instar inoculated batch, second organism on second day, after six hours third organism was inoculated and fourth organism was administered after twelve hours. While in fifth instar inoculated batch, second organism was given on third day of fifth instar (i.e., after 48 hours), third organism was inoculated on fourth day (i.e., after 24 hours) and after six hours fourth organism was inoculated.

Statistical Analysis

The experiment consisted of three replications, 50 worms in each replication. The data was analysed using one way variance (Completely Randomized Design) as per Sundarraj *et al*. (1972) [12].

Results and Discussion

In the present study, the inoculum taken for per oral inoculation of third, fourth and fifth instar inoculated batches consists of 3.16 × 109 CFU/ml (Acinetobacter baumannii), 4.70 × 109 CFU/ml (Bacillus pumilus), 3.07 × 109 CFU/ml (Bacillus licheniformis) and 2.12 × 10-11 PIBs/ ml (CPV) at 10-7 dilution.

Per cent larval weight reduction over sterile water control (%)

Ten larvae of each instars were randomly selected from each treatment and replication wise and larval weight was recorded at the beginning and end of third and fourth instar using electronic balance. Besides, on the fifth day of fifth instar average weight of ten worms from each treatment and the percent larval weight reduction over sterile water control was calculated replication wise.

The statistical data revealed that, significant larval weight reduction over sterile water control increased, as the number of organisms administered to the silkworm increased i.e., 43.50 per cent in (C + A + Bp + Bl) followed by 38.55 per cent in (C + Bp + Bl), 37.54 (C + A + Bp), 30.95 (C + Bl + A), 27.08 (C + Bp), 26.03 (C + Bl), 19.62 (C + A) in third instar inoculated batch.

The same trend was observed in fourth instar of fourth instar inoculated batch where maximum larval weight reduction was noticed in (C + A + Bp + Bl) 37.11 per cent and minimum was registered in (C + A) 28.14 per cent. However, 100 per cent larval weight reduction was observed in fifth instar of the same batch which were not survived till end. The decreasing trend of per cent larval weight reduction which was noticed in fifth instar inoculated treatment combination viz., 34.05 per cent in (C + A + Bp + Bl) followed by 32.66 per cent in (C + Bp + Bl), 30.32 (C + A + Bp), 27.20 (C + Bl + A), 26.07 (C + Bp), 22.73 (C + Bl) and 14.88 (C + A). The reduction in the larval weight over sterile water control revealed maximum per cent larval reduction in mixed infection of CPV with three bacterial combination, followed by dual bacterial combination with CPV and least per cent larval reduction was registered in CPV with single bacterial combination (Table 1).

These experimental results are in confirmative with the findings Anusha and Bhaskar (2016) [2] who reported that the combined effect of NPV and other bacterial isolates decreased larval weight ranged from 0.77 (NPV + Bacillus subtilis + Lysinibacillus sphericus) to 0.83 (NPV + Alcaligenes

faecalis) at the end of third instar, whereas, 0.82 (NPV + Lysinibacillus sphericus + Alcaligenes faecalis) to 0.86 (NPV + Alcaligenes faecalis) and 2.76 (NPV + Bacillus subtilis + Lysinibacillus sphericus) to 3.00 (NPV + Alcaligenes faecalis), 2.89 (NPV + Bacillus subtilis + Lysinibacillus sphericus) to 3.18 (NPV + Alcaligenes faecalis) and 18.65 (NPV + Bacillus subtilis + Lysinibacillus sphericus) to 19.40 (NPV + Alcaligenes faecalis) g/10 at the beginning and end of the fourth and fifth instar over distilled water administered batch (0.99; 1.04 and 4.60; 4.68 and 24.85 g/10 larvae) respectively.

Table 1: Effect of per-oral inoculation of *Bm*CPV with different bacterial isolates on larval weight reduction over sterile water control (%) of silkworm PM×CSR$_2$

Treatment	III instar inoculated					IV instar inoculated			V instar inoculated
	III instar	IV instar		V instar		IV instar	V instar		V instar
	End	Beginning	End	Beginning	End	End	Beginning	End	End
T_1- C + A	15.32 (23.06)	17.16 (24.48)	21.74 (27.81)	20.85 (27.18)	19.62 (26.31)	28.14 (32.05)	27.77 (31.81)	100.00 (90.05)	14.88 (22.70)
T_2- C + Bp	17.60 (24.82)	15.74 (24.52)	35.12 (36.36)	33.60 (35.44)	27.08 (31.38)	33.64 (35.47)	32.26 (34.63)	100.00 (90.05)	26.07 (30.72)
T_3- C + Bl	16.11(23.67)	17.21 (25.58)	30.36 (33.45)	28.34 (32.18)	26.03 (30.69)	31.61 (34.23)	30.74 (33.69)	100.00 (90.05)	22.73 (28.49)
T_4- C + A + Bp	17.99 (25.11)	18.63 (25.58)	36.99 (37.48)	36.56 (37.23)	37.54 (37.81)	35.56 (36.63)	34.62 (36.06)	100.00 (90.05)	30.32 (33.43)
T_5- C + Bp + Bl	16.07 (23.65)	18.28 (25.32)	38.95 (38.63)	38.50 (38.37)	38.55 (38.40)	36.55 (37.22)	35.83 (36.79)	100.00 (90.05)	32.66 (34.87)
T_6- C + Bl + A	17.64 (24.85)	20.39 (26.85)	35.99 (36.88)	35.08 (36.34)	30.95 (33.82)	34.29 (35.86)	34.30 (35.87)	100.00 (90.05)	27.20 (31.45)
T_7- C + A + Bp + Bl	18.40 (25.41)	19.02 (25.87)	40.62 (39.62)	40.65 (39.63)	43.50 (41.29)	37.11 (37.55)	36.37 (35.87)	100.00 (90.05)	34.05 (35.72)
T_8- Normal leaf	3.44 (10.69)	6.11 (14.32)	3.51 (10.81)	1.94 (8.01)	1.09 (6.00)	2.32 (8.76)	1.82 (7.77)	0.58 (4.36)	3.04 (10.04)
T_9- Sterile water	0.00 (0.00)	0.00 (0.00)	0.00 (0.00)	0.00 (0.00)	0.00 (0.00)	0.00 (0.00)	0.00 (0.00)	0.00 (0.00)	0.00 (0.00)
F test	*	*	*	*	*	*	*	*	*
S.Em ±	2.241	2.644	1.679	1.701	3.024	2.050	1.830	0.104	3.175
CD at 5%	6.658	7.857	4.988	5.053	8.986	6.091	5.436	0.308	9.432

Acinetobacter baumannii (A), *Bacillus pumilus* (Bp), *Bacillus licheniformis* (Bl) and CPV (C)

Larval duration (days)

The number of days required for the completion of each instar by 50 per cent of the population was recorded in all the treated lot.

The data on total larval duration revealed a significant variation due to inoculation of CPV with different bacterial isolates at third, fourth and fifth instar inoculated batches. The third instar inoculated larvae noticed more number of larval days for completion of larval duration of all the three instars, *viz*., third, fourth and fifth instar. The batches inoculated with (C + A + Bp + Bl) has taken more number of days to complete their instars (21.55 days), followed by T_6- (21.01), T_5 (20.83), T_7 (20.63), T_3 (20.15), T_4 (19.69) and less number of days to complete their instars was recorded in T_2 (18.88), compared to control (15.83) (Table 2).

The combined effect of CPV along with different bacterial isolates on total larval duration varied in fourth and fifth instar of fourth instar inoculated batch. In fourth instar, significant variation was noticed in all the treatments, but the trend was not same in fifth instar, due to complete mortality of worms before completing fifth instar.

The number of days taken for the completion of larval stage was recorded more in third instar inoculated batch followed by fifth and less number of days were taken in fourth instar inoculated batch due to complete mortality of the fifth instar worms in fourth instar inoculated batch.

These findings were confirmative with Govindan *et al*. (1990) [7] who reported that, the fourth instar larval duration in virus treatment was 4.75 to 5.17 days and in bacterial infection it was 4.50 to 4.83 days, while in control the duration was 4.08 to 4.17 days where as fifth instar larval duration was 10.08 to 10.75, 8.33 to 8.75 and 7 to 7.08 days respectively in virus and bacterial treatment and control. Whereas, in the present experimentation the fourth instar larval duration ranged from 4.85 to 5.96 days and 4.95 to 6.33 days in third and fourth instar inoculated batches. Fifth instar larval duration ranged from 10.00 to 11.11 days and 9.45 to 10.42 days respectively in third and fifth instar inoculated batches.

Table 2: Effect of per-oral inoculation of *Bm*CPV with different bacterial isolates on larval duration (days) of silkworm PM×CSR$_2$

Treatments	III instar inoculated batch				IV instar inoculated batch			V instar inoculated batch
	III	IV	V	Total	IV	V	Total	V
T_1- C+A	3.50	5.16	10.22	18.88	5.05	0.00	5.05	9.52
T_2- C+Bp	4.12	5.33	10.70	20.15	5.32	0.00	5.32	9.99
T_3- C+Bl	4.08	5.26	10.35	19.69	5.23	0.00	5.23	9.96
T_4- C+A+Bp	4.22	5.72	10.89	20.83	6.09	0.00	6.09	10.27
T_5- C+Bp+Bl	4.27	5.79	10.95	21.01	6.28	0.00	6.28	10.34
T_6- C+Bl+A	4.17	5.67	10.79	20.63	5.94	0.00	5.94	10.10
T_7- C+A+Bp+Bl	4.48	5.96	11.11	21.55	6.33	0.00	6.33	10.42
T_8- Normal leaf	3.06	4.18	8.59	15.83	4.08	9.67	13.75	9.08
T_9- Sterile water	3.06	4.18	8.59	15.83	4.08	9.67	13.75	9.00
F test	*	*	*		*	*		*
S.Em ±	0.085	0.089	0.083		0.076	0.021		0.073
CD at 5%	0.251	0.262	0.244		0.224	0.061		0.217

Acinetobacter baumannii (A), *Bacillus pumilus* (Bp), *Bacillus licheniformis* (Bl) and CPV (C)

Moulting duration (hours)

The number of hours required for the completion of each moult by 50 per cent of the population was recorded in all the treated lot.

The data on moulting duration recorded significant results over the control batches in third and fourth instar inoculated batches. The flacherie disease causing pathogens along with CPV altered moulting duration in both third and fourth instar

inoculated batch. However, maximum and minimum moulting duration was registered in T_8- (85.50 and 51.62 h) and T_1- (49.33 and 37.83 h) respectively. The trend found remained same for total moulting duration (hours) in third instar inoculated batch *i.e.*, T_8 recorded maximum of 137.12 hours and T_1 was recorded minimum of 87.16 hours, whereas, the total moulting duration of control batch *viz*., T_9 – normal leaf and T_{10} – sterile water were recorded 52.25 and 52.42 hours, respectively.

Incase of fourth instar inoculated batch, significant variation was found between dual inoculated batches *viz*., T_1 (31.08), T_2- (39.72), T_3- (36.88) and multiple (three to four organisms) inoculated batches *viz*., T_4- (44.30), T_5- (46.90), T_6- (43.72) and T_7- (48.37). Here highest moulting duration was noticed in T_7- (48.37) and least was recorded in T_1- (31.08).

The statistical data interprets that, the moulting duration of the treated larvae of PM×CSR_2 of third, fourth and fifth instar inoculated batches extended significantly over control. More moulting duration was recorded in silkworms treated with four organisms (T_7), followed by three organisms (T_4, T_5 and T_6) and two organisms (T_2, T_3 and T_4). This interprets that, there was synergistic effect of one organism on the other, when inoculated in combination.

This was in parity with Govindan *et al.* (1990) [7] who reported that, the fourth moulting duration was 24 h in control batches, while it prolonged significantly among worms infected with kenchu viral dilutions alone (30 to 32 h) and as well in all the batches of worms infected simultaneously with virus and bacteria (28 to 36 h). The same trend was noticed in the present study, in which the fourth moulting duration was 26.75 and 26.00 hours in sterile water control batches, while it prolonged significantly among worms infected with CPV along with single bacterial isolate (38.37-42.30 and 31.08-39.72 h) followed by CPV with two bacterial isolates (47.67-49.32 and 43.72-46.90 h) and longest moulting duration was recorded in CPV with three bacterial isolates (51.62 and 48.37 h) in third and fourth instar inoculated batches respectively.

In general, the moulting duration taken by third and fourth inoculated batches noticed more time than that of control batches. It may be attributed that, any pathogenic load inside the body of silkworm alter moulting hormone synthesis, as a result there was variation between each instar compared to that of control lot. It may be inferred that, pathogenic groups of organism within the larval body change the physiology of silkworm, may be the right cause for extending moulting duration in all the combination with CPV.

Table 3: Effect of per-oral inoculation of *Bm*CPV with different bacterial isolates on moulting duration (hours) of silkworm PM×CSR_2

Treatments	III instar inoculated batch			IV instar inoculated batch
	III	IV	Total	IV
T_1- C+A	42.57	38.37	80.94	31.08
T_2- C+Bp	45.90	42.30	88.20	39.72
T_3- C+Bl	43.67	40.17	83.84	36.88
T_4- C+A+Bp	51.78	48.58	100.36	44.30
T_5- C+Bp+Bl	52.43	49.32	101.75	46.90
T_6- C+Bl+A	50.25	47.67	97.92	43.72
T_7- C+A+Bp+Bl	55.50	51.62	107.12	48.37
T_8- Normal leaf	25.50	26.92	52.42	26.17
T_9- Sterile water	25.50	26.75	52.25	26.00
F test	*	*		*
S.Em ±	1.143	1.859		1.713
CD at 5%	3.372	5.483		5.053

Acinetobacter baumannii (A), *Bacillus pumilus* (Bp), *Bacillus licheniformis* (Bl) and CPV (C)

Per cent worms entering to subsequent instar (%)

The number of larvae entering to next instar was recorded for all treatments of third, fourth and fifth instar treated lots and then converted to percentage

The experimental data on per cent worms entering to fourth instar was recorded more in T_1 (90.00%) and minimum was noticed in T_7 (42.67%). However, the same trend was followed for per cent worms that entered fifth instar *i.e*., 60.00 per cent in T_1 and 28.00 per cent in T_7 in third instar inoculated batch. The trend of per cent worms entering to subsequent instar *i.e*., from fourth instar to fifth instar in fourth instar inoculated batch revealed that the highest percentage of worms entering fifth instar was recorded in T_1 (68.67%), followed by T_3 (54.67%), T_2 (54.00%), T_6 (50.67%), T_4 (46.00%), T_5 (41.33%), whereas lowest percentage of worms entered to fifth instar was recorded in T_7 (28.67%).

Further Doreswamy (2002) also observed that, inoculation of silkworm larvae with *Bm*IFV (48.22/50), *Bm*DNV (48.33/50), *Bacillus species* (45.66/50), *Streptocaoccus faecalis* (44.22/50) and *Staphylococcus aureus* 44.88/50 individually recorded significantly more number of worms progressed to fifth instar compared to mixed infection with *Bm*IFV+ *Bm*DNV+ *S. aureus* (39.33/50) and *Bm*IFV + *Bm*DNV + *S. faecalis* (39.50/50).

The same trend was seen in the present study where combined inoculation of silkworm with three organisms *viz*., T_4 - CPV + *Acinetobacter baumannii* + *Bacillus pumilus* (52.00 and 46.00%), T_5- CPV + *Bacillus pumilus* + *Bacillus licheniformis* (46.00 and 41.33%), T_6- CPV + *Bacillus licheniformis* + *Acinetobacter baumannii* (52.67 and 50.67%) followed by administration with four organisms *i.e*., T_7- CPV + *Bacillus licheniformis* + *Bacillus pumilus* + *Bacillus licheniformis* (42.67 and 28.67%) percentage of worms entered to fourth instar and fifth instar in third and fourth instar inoculated batch respectively.

Table 4: Effect of per-oral inoculation of *Bm*CPV with different bacterial isolates on per cent worms entering to subsequent instar (%) of silkworm PM×CSR_2

Treatments	III instar inoculated batch			IV instar inoculated batch	
	III	IV	V	IV	V
T_{1}- C+A	100	90.00	60.00	100	68.67
T_{2}- C+Bp	100	53.33	40.00	100	54.00
T_{3}- C+Bl	100	69.33	40.67	100	54.67
T_{4}- C+A+Bp	100	52.00	34.00	100	46.00
T_{5}- C+Bp+Bl	100	46.00	31.33	100	41.33
T_{6}- C+Bl+A	100	52.67	38.00	100	50.67
T_{7}- C+A+Bp+Bl	100	42.67	28.00	100	28.67
T_{8}- Normal leaf	100	100.00	97.33	100	100.00
T_{9}- Sterile water	100	100.00	98.00	100	100.00
F test	NS	*	*	NS	*
S.Em ±	-	3.341	2.694	-	2.740
CD at 5%	-	9.926	8.005	-	8.140

Acinetobacter baumannii (A), *Bacillus pumilus* (Bp), *Bacillus licheniformis* (Bl) and CPV (C)

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