

PER ORAL INOCULATION OF *LYSINIBACILLUS SPHAERICUS* WITH PATHOGENIC MICROBES ON REARING AND COCOON PARAMETERS OF SILKWORM, *Bombyx mori* L.

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ABSTRACT

Mixed infections are complex interactions between pathogens and silkworm which cause diseases and reduces cocoon crop loss. The per oral inoculation of pathogenic bacterium *Lysinibacillus sphaericus* along with viz., *A. faecalis* (A), *B. subtilis* (B) and NPV (N) to third instar PM×CSR₂ (50 silkworms/ replication) resulted minimum of 7.87 and 9.85 days for ET₅₀ symptom expression and mortality in treatment T₆- *L. sphaericus* + *A. faecalis* + NPV. The remaining treatments T₅ triple inoculation (L+A+B) (9.89 and 12.36 days) and dual inoculation T₂ (L+A) (20.89 and 23.29 days), T₃ (L+B) (10.10 and 12.48 days) and T₄ (L+N) (10.18 and 11.18 days) recorded different days for symptom expression and mortality. Further, number of larvae entered to fourth instar was noticed minimum in T₆-*L. sphaericus* + *A. faecalis* + NPV (30) and maximum in T₂-*L. sphaericus* + *A. faecalis* (44). Further, the cocoon parameters viz., single cocoon weight (1.00 g), shell weight (0.08 g), shell ratio (8.00) and silk productivity (0.69 cg/ day) was noticed minimum in T₆. The interaction effect of *Lysinibacillus sphaericus* with other pathogenic bacteria viz., *Bacillus subtilis*, *Alcaligenes faecalis* and NPV had synergistic effect compared to *Lysinibacillus sphaericus* alone.

INTRODUCTION

Among different diseases, the bacterial Flacherie is considered to be a major disease in silkworm and flaccidity of larva is the major symptom. The disease is very common during summer and rainy seasons in all the sericultural areas of India which accounts to crop loss of 27 – 35% with cocoon yield loss of 11 – 15kg /100 dfls. It is reported that, flacherie is caused by different species of bacteria, viruses and their mixed infections (Selvakumar, 2013). In most of the cases of silkworm rearing, the bacterial flacherie disease is caused by involvement of different groups of pathogenic bacteria and virus. Therefore, these bacteria many a times have mutualistic effect and very rarely they are pathogenic during their occurrence in silkworm larva and affect the cocoon parameters (Anusha and Bhaskar et al., 2016). In this view, flacherie disease samples were collected from farmers rearing house in Kolar and Chikkaballapur districts of Karnataka and among isolated strains, commonly found three strains of bacteria (B1, B2 and B3) were identified through molecular techniques as *Lysinibacillus sphaericus* (L), *Alcaligenes faecalis* (A), *Bacillus subtilis* (B) (Anusha et al., 2016) and experiment was carried out to know the 'Interaction effect of *Lysinibacillus sphaericus* with pathogenic microbes on rearing and cocoon parameters of silkworm, *Bombyx mori* L.

MATERIALS AND METHODS

To know the interaction effect of per oral inoculation of *Alcaligenes faecalis*, *Lysinibacillus sphaericus*, *Bacillus subtilis* and NPV was carried out. Immediately after the third moult of

Pure Mysore ×CSR₂, silkworms were inoculated with the bacteria (at the rate of 0.25 ml per 25 larvae with 10⁻⁷ dilution to each replication at the beginning of the third instar) by smearing the bacterial solution onto the leaf surface, dried in shade. While inoculating two micro-organisms, first micro-organism was inoculated at the beginning of the third instar and the second organism (isolate) was inoculated during middle of the third instar. In case of three Micro-organism administration, first one micro-organism was administered at the beginning and the other two Micro-organisms were inoculated at the middle of the third instar larvae of PM×CSR₂ (Govindan et al., 1998). Same number of larvae were fed with distilled water smeared mulberry leaves and normal mulberry leaf which was considered as a control. Silkworm larvae were reared at 27°C room temperature with relative humidity 70 - 80% (Suparna et al., 2011).

Treatments details:*Lysinibacillus sphaericus* with other organisms

T₁- *Lysinibacillus sphaericus*

T₂- *Lysinibacillus sphaericus*+ *Alcaligenes faecalis*

T₃- *Lysinibacillus sphaericus*+ *Bacillus subtilis*

T₄- *Lysinibacillus sphaericus*+ NPV

T₅- *Lysinibacillus sphaericus* + *Alcaligenes faecalis* + *Bacillus subtilis*

T₆- *Lysinibacillus sphaericus* + *Alcaligenes faecalis* + NPV

T₇- Normal Leaf

T₈- Sterile water

The rearing and cocoon parameters were recorded for

evaluation *viz.*, larval weight reduction, larval duration, moulting duration, ET₅₀ for symptom expression and mortality, number of silkworms entering to subsequent instar and spinning. ERR, larval mortality, single cocoon weight, single shell weight, Shell ratio and silk productivity and one way anova data analysis was used for statistical analysis (Sundarraj *et al.*, 1972).

RESULTS AND DISCUSSION

The experimental data on combined inoculation of *Lysinibacillus sphaericus* with other organisms registered significant results on per cent weight reduction over control was noticed in third instar inoculated larvae of PM×CSR₂.

The maximum per cent larval weight reduction of 23.56, 41.06, 23.94 were observed at the end of third, fourth and fifth instar

larvae of PM×CSR₂ administered with T₆-*Lysinibacillus sphaericus* + *Alcaligenes faecalis* + NPV and minimum (16.10, 16.06 and 15.48 %) was found during the inoculation of single pathogen (T₁-*Lysinibacillus sphaericus*). Further, among dual inoculation of bacterial isolates more larval weight reduction was found in T₄-*Lysinibacillus sphaericus* + NPV (20.14, 29.96 and 19.55 %) followed by T₃-*Lysinibacillus sphaericus* + *Bacillus subtilis* (17.09, 26.44, 19.15 %) and T₂-*Lysinibacillus sphaericus* + *Alcaligenes faecalis* (15.12, 23 and 11.50 %) over sterile control (Table 1).

The experimental data, on total larval duration as well as moulting duration registered significant variation. However, maximum (5.38, 6.98 and 11.85 days) and minimum of 4.80 5.98 and 9.98 days were recorded during third, fourth and fifth instars of inoculated batches of T₅-*Lysinibacillus sphaericus* + *Alcaligenes faecalis* + *Bacillus subtilis* and T₁-

Table 1: Influence of combined inoculation of *Lysinibacillus sphaericus* with other different bacterial isolates on rearing parameters of PM×CSR₂

Treatments	Larval weight reduction (%)			Larval duration (Days)				Moulting duration (h)			ET ₅₀ Symptoms (Days)	ET ₅₀ Mortality (Days)
	III instar End	IV instar End	V instar End	III	IV	V	Total	III	IV	Total		
T ₁ -L	16.1 (23.53)	16.06 (23.6)	15.48 (23.14)	4.8	5.98	9.98	20.76	30.96	31.1	62.06	11.57	14.1
T ₂ -L+A	15.12 (22.74)	23 (28.64)	11.5 (19.78)	4.9	6.1	10.29	21.29	32.28	33.22	65.5	20.89	23.29
T ₃ -L+B	17.09 (24.33)	26.44 (30.51)	19.15 (25.95)	5.18	6.24	10.56	21.98	34.18	35.96	70.14	10.1	12.48
T ₄ -L+N	20.14 (26.62)	29.96 (34.23)	19.55 (26.24)	5	6.18	10.98	22.16	31.68	32.18	63.86	10.18	11.18
T ₅ -L+A+B	17.13 (24.36)	37.38 (33.79)	19.11 (25.92)	5.38	6.98	11.85	24.21	39.96	40	79.96	9.89	12.36
T ₆ -L+A+N	23.56 (28.96)	41.06 (39.85)	23.94 (29.29)	5.21	6.64	11.45	23.3	36.8	37.1	73.9	7.87	9.85
T ₇ -Control	3.03 (10.02)	2.2 (7.07)	0.8 (5.13)	3.16	4.28	6.98	14.42	26.1	26.2	52.3	0	0
T ₈ -SW	0 (0.58)	0 (0.58)	0 (0.58)	3.16	4.28	6.98	14.42	26.1	26.2	52.3	0	0
F-test	*	*	*	*	*	*		*	*		*	*
SEm ±	2.77	1.5	0.85	0.04	0.02	0.07		0.04	0.3		0.58	1
CD at 5 %	5.88	3.18	1.8	0.08	0.05	0.16		0.09	0.63		1.23	2.11

* - Significant at 5 %; T₁-*Lysinibacillus sphaericus*(L), T₂-*Lysinibacillus sphaericus* + *Alcaligenes faecalis* (L + A), T₃-*Lysinibacillus sphaericus* + *Bacillus subtilis* (L + B), T₄-*Lysinibacillus sphaericus* + NPV (L + N), T₅-*Lysinibacillus sphaericus* + *Alcaligenes faecalis* + *Bacillus subtilis* (L + A + B), T₆-*Lysinibacillus sphaericus* + *Alcaligenes faecalis* + NPV (L + A + N), T₇-Normal Leaf (Control), T₈-Sterile water (SW)

Table 2 : Rearing parameters of PM×CSR₂ as influenced by *Lysinibacillus sphaericus* with other bacterial isolates causing flacherie disease of silkworm, *Bombyx mori*(Third instar inoculated batches)

Treatments	III instar inoculated batches			No. of worms entering to spinning (%)	Effective rate of rearing (%)	Larval mortality (%)
	No. of worms entering into subsequent instars					
	III	IV	V			
T ₁ -L	50	40	28	42.04	37.97	62.03
T ₂ -L+A	50	44	34	56.1	47.95	52.05
T ₃ -L+B	50	38	26	36.03	31.8	68.2
T ₄ -L+N	50	32	24	32.2	29.75	70.25
T ₅ -L+A+B	50	34	25	38.03	27.93	72.07
T ₆ -L+A+N	50	30	21	30.04	19.71	80.29
T ₇ -Control	50	49	49	96.05	91.7	8.3
T ₈ -SW	50	49	49	96.08	93.97	6.03
F-test	NS	*	*	*	*	*
SEm ±	2.76	1.38	1.08	1.68	0.95	1.36
CD at 5 %	5.86	2.93	2.28	3.56	2.02	2.9

* - Significant at 5 %, NS - Non significant; T₁-*Lysinibacillus sphaericus*(L), T₂-*Lysinibacillus sphaericus* + *Alcaligenes faecalis*(L + A), T₃-*Lysinibacillus sphaericus* + *Bacillus subtilis* (L + B), T₄-*Lysinibacillus sphaericus* + NPV (L + N), T₅-*Lysinibacillus sphaericus* + *Alcaligenes faecalis* + *Bacillus subtilis* (L + A + B), T₆-*Lysinibacillus sphaericus* + *Alcaligenes faecalis* + NPV (L + A + N), T₇-Normal Leaf (Control), T₈-Sterile water (SW)

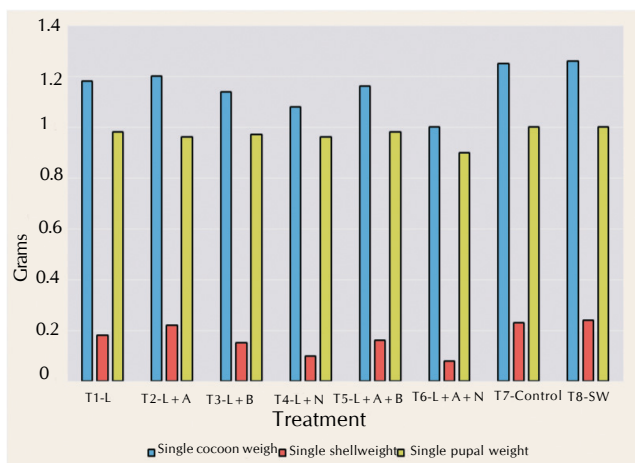


Figure 1: Deterioration of cocoon parameters as influenced by Lysinibacillus sphaericus with other bacterial isolates causing flacherie disease of silkworm, Bombyx mori

Table 3: Deterioration of cocoon parameters as influenced by Lysinibacillus sphaericus with other bacterial isolates causing flacherie disease of silkworm, Bombyx mori (Third instar inoculated batch)

Treatments	III Instar inoculated batch				
	Single cocoon weight (g)	Single shell weight (g)	Single pupal weight (g)	Shell ratio (%)	Silk productivity (cg/day)
T ₁ -L	1.18	0.18	0.98	15.25	1.74
T ₂ -L+A	1.2	0.22	0.96	18.33	1.2
T ₃ -L+B	1.14	0.15	0.97	13.15	1.42
T ₄ -L+N	1.08	0.1	0.96	9.25	0.9
T ₅ -L+A+B	1.16	0.16	0.98	13.79	1.35
T ₆ -L+A+N	1	0.08	0.9	8	0.69
T ₇ -Control	1.25	0.23	1	18.4	3.29
T ₈ -SW	1.26	0.24	1	19.05	3.43
F-test	*	*	*	*	*
SEm±	0.03	0.02	0.05	0.91	0.06
CD at 5 %	0.06	0.06	0.11	1.93	0.13

* - Significant at 5 %, NS - Non significant; T₁-Lysinibacillus sphaericus(L), T₂-Lysinibacillus sphaericus + Alcaligenes faecalis(L+A), T₃-Lysinibacillus sphaericus + Bacillus subtilis (L+B), T₄-Lysinibacillus sphaericus + NPV (L+N), T₅-Lysinibacillus sphaericus + Alcaligenes faecalis + Bacillus subtilis (L+A+B), T₆-Lysinibacillus sphaericus + Alcaligenes faecalis + NPV (L+A+N), T₇-Normal Leaf (Control), T₈-Sterile water (SW)

Lysinibacillus sphaericus respectively. It is very clearly indicated that, administration of T₁-Lysinibacillus sphaericus alone recorded less larval duration compared to T₂- Bacillus subtilis + Lysinibacillus sphaericus (4.90, 6.10 and 10.29), T₃- Lysinibacillus sphaericus + Bacillus subtilis (5.18, 6.24 and 10.56) and T₄- Lysinibacillus sphaericus + NPV (5, 6.18, and 10.98) days which differed significantly compared to inoculation of T₆- Bacillus subtilis + Lysinibacillus sphaericus + NPV (5.21, 6.64 and 11.45 days). Further, the maximum of 39.96 and 40.00 hours and minimum of 30.96 and 31.10 hours of moulting duration was recorded for third and fourth moult of third instar inoculated batch (Table 1).

The inoculated batch with Lysinibacillus sphaericus registered non significant results with T₃-Lysinibacillus sphaericus + Bacillus subtilis (10.10), T₄-Lysinibacillus sphaericus + NPV (10.18) and T₅-Lysinibacillus sphaericus + Alcaligenes faecalis + Bacillus subtilis (9.89) days where as significant difference in expression of disease symptom with T₁-Lysinibacillus sphaericus (11.57) alone, T₂-Lysinibacillus sphaericus +

Alcaligenes faecalis (20.89), T₃-Lysinibacillus sphaericus + Bacillus subtilis (10.10), T₅- Lysinibacillus sphaericus + Alcaligenes faecalis + Bacillus subtilis (9.89) and T₆-Lysinibacillus sphaericus + Alcaligenes faecalis + NPV (7.87) days for ET50 symptom expression value which are found to be highly pathogenic compared to dual administration. The bacterial combination with virus, there was decrease in ET50 value for mortality the minimum was 9.85 days in T₆-Lysinibacillus sphaericus + Alcaligenes faecalis + NPV (Table 1).

In addition significant variation existed with respect to number of worms entering into subsequent instar, among dual administration of Lysinibacillus sphaericus with Alcaligenes faecalis more number of silkworms entered to next instars (50 to 44, 44 to 34) followed by with Bacillus subtilis (50- 38, 38-26) and with NPV (50-32, 32-24), whereas during triple inoculation of Lysinibacillus sphaericus with Alcaligenes faecalis+Bacillus subtilis (50-34, 34-25) and Alcaligenes faecalis+ NPV (50-30, 30-21) resulted less compared to control (50-49, 49-49). The trend was same with respect to number of worms entering to spinning was same (Table 2).

Further, effective rate of rearing was recorded less in treatment T₆-Lysinibacillus sphaericus + Alcaligenes faecalis + NPV(19.71%) and maximum in T₂-Lysinibacillus sphaericus + Alcaligenes faecalis (47.95%) where as per cent larval mortality more in T₆-Lysinibacillus sphaericus + Alcaligenes faecalis + NPV (80.29 %) and minimum in T₂-Lysinibacillus sphaericus + Alcaligenes faecalis (52.05 %) because of interaction of two different micro organisms resulted more larval mortality due to negative effect (Table 2).

The interaction effect of Lysinibacillus sphaericus with other organisms resulted variation in single cocoon weight, shell weight, shell ratio and silk productivity. The maximum and minimum cocoon weight, shell weight, shell ratio and silk productivity were noticed in T₂-Lysinibacillus sphaericus + Alcaligenes faecalis (1.20, 0.22, 18.33 % and 1.2 cg/day) and T₆-Lysinibacillus sphaericus + Alcaligenes faecalis + NPV (1.00 g., 0.08 g., 8.00 % and 0.69 cg/day) compared to control (1.26, .24 g, 19.05 % and 3.43 cg/day) (Table 3)(Figure 1).

These findings are in confirmative with Chitra et al. (1974) different bacterial isolates viz., Aerobacter cloacae, Achromobacter superficialis, Achromobacter delmarvae, Staphylococcus albus, Escherichia freundii and Pseudomonas ovalis varied in their pathogenicity to silkworms and stage of growth of larvae affected their virulence. Invasion of pathogens into larval body caused considerable reduction in larval weight from 10.10 to 27.50 per cent in V instar of silkworm, B. mori and deleterious effect increased with age of infection. It is further attributed that, per oral inoculation of Bacillus species to PM×CSR2 resulted in decreased larval weight from the beginning and end of the fourth instar (1.95-13.40 g/ 10 in Pure Mysore, 5.40-21.5 g/10 in CSR2) compared to control(2.55-13.80 and 6.38-23.5) (Anusha and Bhaskar, 2016).

Further, Doreswamy (2002) observed that, when Bacillus sp., Streptococcus faecalis and Staphylococcus aureus were administered to fourth instar larvae of PM×NB4D2 hybrid significantly longer duration of 114.88, 119.66, 123.05 h was noticed compared to control 102.00 h. and Manoj et al. (2013)

reported that, moulting was delayed by nearly 24 h between inoculated groups and control, when the bacterial suspensions of 1.0×10^6 CFU and 4.0×10^6 CFU was inoculated to the silkworm larvae.

The oral administration of *Staphylococcus* sp. and *Serratia marcescens* were cause variation in worms entering to spinning, when third, fourth and fifth instar larvae of PM \times C. Nichi were fed at the rate of 10×10^6 , 1×10^6 and 10^6 cell/ larvae revealed 45/50, 38/50 50/50 entered to spinning (Vasantharajan and Munirathamma, 1978).

Selvakumar *et al.* (2009) studied on the synergism between the pathogen *viz.*, *Streptococcus* sp. and BmDENV1 where *Streptococcus* sp. bacteria caused 6.00 (low) and 31.67 per cent (medium) mortality in silkworm. The mortality was initiated on 11th and 10th day post inoculation with low and medium pathogenic bacteria, respectively. However, BmDENV1 alone in different concentrations ($IC_{50} \times 10^2$, $IC_{50} \times 10^1$, IC_{50} and $IC_{50} \times 10$) did not cause any mortality. BmDENV1 in synergistic association with low pathogenic *Streptococcus* led to increased mortality from 6.00 to 39.33-64.76 per cent. The mortality was also advanced from 11th day to 6th day. BmDENV1 in synergistic association with medium pathogenic *Streptococcus* also led to increase in mortality from 31.67 to 85.33-95.33 per cent. The mortality was advanced from 10th to 5th day post inoculation.

In addition, Rosa Estela Quiroz *et al.* (2015) reported, the supernatant extract (1 μ g) of *A. faecalis* MOR02 killed more than 70 per cent *G. mellonella* larvae 96 h after infection. Further, when the larvae were injected with 240 CFU and 2,400 CFU had 3.33 per cent mortality at 72 h after injection and 6.67 per cent at 48 h after injection, respectively. With 2,400 injection CFU, 33.33 per cent mortality was observed at 96 h after injection. Conversely, larvae injected with 24,000 CFU had 96.67 per cent mortality at 24 h after injection, while 100 per cent mortality was observed using 2,40,000 CFU.

The effect of synergism between *Streptococcus* sp. bacteria and BmDENV1 on single cocoon weight, single shell weight and shell ratio percentage were elucidated by (Selvakumar *et al.*, 2009). The lowest cocoon weight (1.102 g), single shell weight (0.123 g) and shell ratio percentage (11.16 per cent) were noticed in BmDENV1 (IC_{50}) + medium pathogenic bacteria *Streptococcus* sp. (1×10^7) followed by medium pathogenic bacteria *Streptococcus* sp. (1×10^7) + BmDENV1 (IC_{50}) (1.181 g, 0.170 g and 14.39 %) respectively, whereas control batches recorded highest for all the above mentioned parameters.

Shorter filament length was obtained in inoculation with BmIFV + BmDENV (420.27 m), BmIFV + BmDENV + *Bacillus* sp. (424.44 m), BmIFV + BmDENV + *S. faecalis* (426.21), BmIFV + BmDENV + *S. aureus* (445.55 m), BmIFV + *S. aureus* (447.16 m), BmIFV + *S. faecalis* (450.44 m), BmIFV + *Bacillus* sp. (446.10 m), BmDENV + *Bacillus* sp. (442.99 m) and BmDENV + *S. faecalis* (452.21 m) whereas, significantly longer filament length was recorded in distilled water control (730.00 m) and

in untreated control (725.00 m). Significantly thinner denier was obtained in inoculation with BmIFV + BmDENV + *Bacillus* sp. (1.75) and BmIFV + BmDENV (1.80) whereas thicker denier was obtained in distilled water control (2.40) and untreated control (2.30) (Doreswamy *et al.*, 2004).

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