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Antagonistic effects of rhamnolipid on larval gut microbes and impact on the growth and development of the silkworm

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ABSTRACT

The demand for bio-based insecticides has significantly increased during the last decade due to the contamination of the environment following massive pesticide use. The present study aims at evaluating the insecticidal activity of rhamnolipid (RL) biosurfactant (from 0.01 to 1 %) orally administered to fifth instar larvae of the silkworm, *Bombyx mori*, a model among Lepidoptera. The bioassay showed that above 0.5% RL caused significant mortality in the larvae and pupae of *B.mori*. To evaluate the mode of action of RL, we carried out antibacterial activity assays against larval gut microbes of *B.mori*. Interestingly, a fall of the microbial population and a susceptibility of host beneficial bacterial species, like *Bacillus megaterium, Bacterium IMBL3, Enterococcus mundtii* and *Bacillus cerus,* towards RL were observed by using plate sensitive assay and agar well diffusion assay, respectively. The results of the present study confirm that RL cause the breakdown of beneficial interactions between the larval gut and microbes, leading to a high rate of larval mortality due to the digestive upset in the *B. mori* gut. This work provides a platform of knowledge to set up promising strategies for the biological control of insect pests.

Keywords: Rhamnolipid, antibacterial activity, *Bombyx mori,* insecticidal activity, gut microbes.

INTRODUCTION: Rhamnolipid (RL) is well-studied glycolipids that exhibit low toxicity and high biodegradability, making them eco-friendly surfactants. RL are classified as mono- and di-rhamnolipid with excellent surface activity, emulsifying activity and emulsion stability at extremes of temperature, pH, and salinity (Irfan-Maqsood and Seddiq-Shams, 2014). These biosurfactants are mainly produced by microorganisms by using renewable carbon sources. Moreover, these compounds are excellent substitutes for chemical surfactants due to their similar physico-chemical properties (Banat *et al.*, 2014). Many reports discussed about the importance of biosurfactants in the field of agriculture, pharmaceutical, food, cosmetics and detergent industry (Singh and Rale, 2022). The anti-bacterial, fungal, virus and adhesive activities of RL against pathogens are making them useful for treating many diseases. Moreover, they are used as therapeutic and probiotic agents in medical industry (Kumar *et al.*, 2021). The antibacterial activity of RL has been studied by using different analytical techniques, such as UV-Spectroscopy, FTIR and SEM (Bharali *et al.*, 2013). The biological activity of rhamnolipid might be due to the presence of glycolipids with one or two rhamnose molecules linked to HAA fatty acid and their complex structure has been widely described (Ochsner *et al.*, 1994).

The RL is also considered potent agents to substitute synthesized pesticides (Sachdev and Cameotra, 2013). In this context, the exclusive application of chemical insecticides in agriculture to increase crop productivity, as well as to maximize insect pest control, has led to several comprehensive issues including environmental contamination, destruction of natural enemies and, most importantly, development of resistance to the chemicals (Aktar *et al.*, 2009). On the other hand, the increase of prices of new chemical pesticides stimulated the search for new eco-friendly tools for insect control (Boethling *et al.*, 2014). A decade ago, researchers started to evaluate the insecticidal activity of microbial biosurfactants (Awada *et al.*, 2011). Later, Kim *et al.* (2011) investigated the use of rhamnolipid produced by *Pseudomonas aeruginosa* as an insecticidal agent against the green peach aphid, *Myzus persicae*. These microbial metabolites are also called as antifeedant works efficient against *Rhyzoperta dominica and Spodoptera litura,* which attacks stored grains and vegetables respectively (Kama *et al.*, 2012). They also suggest that RL is a natural plant protection agent and ecologically acceptable due to their nature. Silva *et al.* (2015) reported the potential larvicidal, insecticidal and repellent activities of rhamnolipid against *Aedes aegypti*. However, the mechanisms that underpin the insecticidal activity of pest mediated by RL still remain unclear (Kama *et al.*, 2012).

OBJECTIVES: The present study was undertaken to fill in in this gap of knowledge by evaluating the mode of insecticidal activity of rhamnolipid, harvested from *Pseudomonas aeruginosa* DS10-129 against the larvae of the lepidopteran insect model, *Bombyx mori*.

MATERIAL AND METHODS: **Extraction of rhamnolipid:** Rhamnolipid biosurfactant was extracted and concentrated from the culture supernatant of *P. aeruginosa* DS10-129 grown on mineral salts medium supplemented with 1% (v/v) glycerol. A rhamnolipid fraction from the culture-free

supernatant was extracted by adding an equal volume of 2:1 chloroform/methanol solvent mixture and mixing thoroughly. The organic layer was subsequently separated by using a separating funnel, air-dried, and dissolved in methanol. Mass spectrometry characterization and detection of the rhamnolipid fractions under investigation was performed with an LCQ quadrupole ion-trap mass spectrometer utilizing electrospray ionization (Bondarenko *et al.*, 2010). The purified rhamnolipid biosurfactant (15%) was diluted in deionized water and different concentrations of RL were prepared (0.01, 0.1, 0.25, 0.5 and 1%).

Experimental insects: Eggs of *B. mori* (Tamil Nadu White x NB4D2) were purchased from the Government Grain Center (Tiruchirapalli, India) and maintained at 27 ± 2 ˚C with a relative humidity of 75 ± 5% (Nirmala *et al.*, 1999). Hatched larvae were fed with chopped tender leaves of mulberry variety (MR2) until third instar and with coarse leaves until the end of the last larval instar. Bed cleaning and spacing of larvae in trays were performed as described by Vanishree *et al.* (2005).

Bioassays: Mulberry leaves were washed with double distilled water, dipped in different concentration of RL solutions and semidried under fan. RL-treated leaves were fed to *B. mori* larvae (L5D3; fifth larval instar day 3). The RL treatment was carried out a single time per day. Each experiment was done in triplicates. The characteristics of the larvae following RL treatment were followed up to adult stage. These included: larval-adult mortality, longevity and eggs laid by mature insects. Data were statistically analyzed by one-way analysis of variance (ANOVA) and significance between control and RL treatment was evaluated with a Tukey's pair-wise comparison using PAST version 3.05 (Hammer, 2001). Data are presented as the mean ± standard deviation (SD). Differences were considered significant if $P < 0.05$.

Microbe isolation from the gut and plate sensitive assay: The *B. mori* larvae (L5D3) were surface sterilized with 70% ethanol prior to dissection for the prevention of microbiological cross-contamination while collecting the gut. The gut was washed with double distilled water, homogenized in 1x PBS (pH 7.4) and sonicated for 30 seconds at 60 Hz to free the microbes from the gut of lumen (Vanishree *et al.*, 2005). To enumerate the gut microbes against RL, the gut suspensions were serially diluted (10^{-1} and 10^{-2}) and then were cultured on Tryptone Soya Agar (TSA) plates supplemented with 0.5 and 1% RL (RL was added after autoclaving TSA medium). Control plates did not contain RL. The plates were incubated overnight at 37 °C. The morphology of the colony was examined (Bergey, 2001) and CFUs were counted using a Digital Colony Counter. This experiment was repeated three times.

Agar well diffusion assay: Antimicrobial activity was determined based on Minimal Inhibitory Concentration (MIC) values, defined as the lowest concentration of the antibacterial agent needed to inhibit the development of visible growth after incubation for the required time. Agar well diffusion assay was performed as described by Bharali *et al.* (2013). In the present study, we selected four bacterial strains [*B. megaterium* (KF951539), *Bacterium IMBL3* (HQ419191), *Enterococcus mundtii* (HQ419189), and *B. cereus* (JN559873)] which were already identified from gut of *B. mori* by using 16S rRNA analysis and submitted to the National Centre for Biotechnology Information (NCBI), USA. These strains were cultured on nutrient broth and incubated overnight at 37 °C in a shaker at 220 rpm. About 100 µL of each bacterial culture was spread on LB agar plates. Approximately 6 mm diameter wells were made in LB agar plate with a sterile steel borer and different concentrations of RL (0.1, 0.25, 0.5, and 1%) were loaded onto the wells (50 µl/well). The antibiotic kanamycin (50µg/mL) was used as a positive control. The plates were incubated overnight at 37 ºC for and the zone of inhibition was measured using a transparent metric ruler (Himedia, India). Experiments were conducted in triplicate.

RESULTS AND DISCUSSIONS: Effects of rhamnolipid on the growth of *B. mori*: Controls and larvae treated with less than 0.5 % RL doses did not show any changes in the feeding behavior. After 2 days of the treatment with RL (0.5 and 1%), larvae became sluggish and stopped feeding, it may be due to antifeedant activity of RL as reported by Kama *et al.* (2012). Similarly, they have also reported the insect pest (*S.litura* and *R.dominica*) showed dose dependent antifeedant activity against RL-1 and RL-2. Bernays *et al.* (2000) reported that RL treatment leads to reduction in food consumption (stop feeding) in the present study might be due post ingestion feedback. In addition, RL treated silkworm larvae showed several pathological symptoms, such as loss of appetite, sluggishness, slow growth and appearance of brown color on the skin (figure 1). Similarly, these kinds of symptoms were observed in silkworms infected by bacteria (Samson, 1995).

Figure 1: : Insecticidal activity of rhamnolipid on *B. mori* larvae (arrows indicate the dead larvae).

The effects of RL on the growth characteristics of treated larvae are shown in Table 1. Interestingly, larval mortality was found at 0.5 and 1% RL ($F=258.7$, $P < 0.05$). Similarly, Kim *et al.* (2011) reported high mortality due to the effects on the gut and the cuticle, after RL exposure, in *M. persicae*. One-way ANOVA with Tukey's pair wise comparison analysis clearly revealed that larval longevity, as well as mortality and longevity of spinning larvae, were not significantly affected by all the concentrations of RL. However, there was a significant increase of pupal mortality ($F=1056$, $P < 0.05$) and a decrease of pupal longevity ($F=5.333$, $P < 0.05$) after treatment with 1% RL. Furthermore, no adults emerged from larvae treated with 1% RL ($F=1.07$, $P < 0.05$). The adults emerged from larvae treated with 0.5% RL produced less number of eggs compared to controls and the larvae treated with less than 0.5% ($F=1.81$, P

<0.05). Kannan *et al.* (2016) reported that decreased level of protein synthesis and less availability of nutrients in the electron beam irradiated larvae of *B.mori* due to oxidative stress caused a significant fall of eggs. Similarly, due to the antimicrobial activity of RL leads to reduction in food digestion in gut of silkworm larvae causes nutritional deficiency affected the the eggs development.

Developmental Stage	Growth characteristics (mean ± standard deviation (SD)†	Concentration of Rhamnolipid (%)						One-way ANOVA	
		Control	0.01	0.1	0.25	0.5	1.0	*F*	*P*
V instar larva	Mortality	0 **a**	0 **a**	0 **a**	0 **a**	14 ± 3.6 **b**	50 ± 5 **c**	258.7	<0.05
	Longevity in days	5 ± 0 **a**	5 ± 0 **a**	5 ± 0 **a**	5 ± 0 **a**	5 ± 0 **a**	5 ± 0 **a**	0	NS
Spinning stage	Mortality	0 **a**	0 **a**	0 **a**	0 **a**	0 **a**	0 **a**	0	NS
	Longevity (days)	2 ± 0 **a**	2 ± 0 **a**	2 ± 0 **a**	2 ± 0 **a**	2 ± 0 **a**	1 ± 0.5 **b**	0.1667	NS
Pupa	Mortality	0 **a**	0 **a**	0 **a**	0 **a**	30 ± 2 **b**	98 ±1 **c**	1056	<0.05
	Longevity (days)	7 ± 0 **a**	7 ± 0 **a**	7 ± 0 **a**	7 ± 0 **a**	7 ± 0 **a**	3 ± 0 **b**	5.333	<0.05
Emergence of adults		100 ± 0 **a**	100 ± 0**a**	100 ± 0 **a**	100 ± 0 **a**	72±3.0 **b**	0.00 **c**	1.07	<0.05
No. of eggs produced/mature insect		57 ± 0.3 **a**	623± 2.5 **b**	647 ± 8 **c**	638 ± 2.5 **d**	599 ± 4.7 **e**	0.00 **f**	1.81	<0.05

Table 1: Effects of rhamnolipid on the developmental stages of Bombyx mori.

in gut of silkworm larvae causes nutritional deficiency affected the the eggs development.

Antimicrobial and antibacterial properties of rhamnolipid: Kama *et al.* (2012) reported that RL has antifeedant activity; however, mode of action on insecticidal activity on insect pest is unclear. Therefore, the present study was carried out to assess the insecticidal activity of RL mediated by their *in vivo* antimicrobial activity on the microbiota of the silkworm gut. The symbiotic relationship between microbes and the insect gut plays a major role in insect survival, growth, nutrition and digestion (Kannan *et al.*, 2019). The bacterial strains from the larval gut of *B. mori* are highly involved in the digestion of leaf materials that contain cellulose, xylan and pectin by the production of microbial enzymes (Prem Anand *et al.*, 2010). The gut suspensions (10^{-1} and 10^{-2}) were applied on Tryptone Soya Agar (RL-TSA) plates supplemented with RL. The doses of RL (0.5 and 1%) were selected for plate sensitive assay based on the insecticidal data shown above.

The plate sensitive assays showed fewer number of microbial colonies (CFU/mL) than control. For the 10^{-1} dilution, they were 1758, 1448 and 762 for control, 0.5 and 1%, respectively. At the same time, the 10^{-2} dilution showed 1220, 128 and 54 CFU/mL for control, 0.5 and 1%, respectively (figure 2).

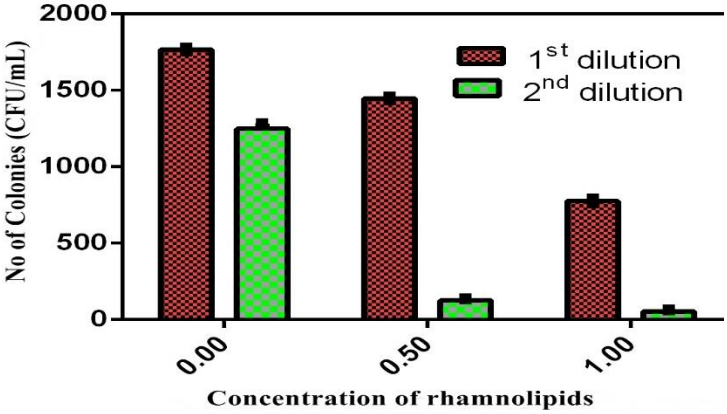

Figure 2: Graphical representation of colony forming units (CFU) of gut microbes (10^{-1} and 10^{-2}) after rhamnolipid treatment using plate sensitivity assay.

Similarly, Subramanian *et al.* (2009) observed that the oral administration of chloromycetin caused a reduction of gut microbiota of silkworms, and this affected the physiology of the insect. In addition, to demonstrate the antimicrobial activity of RL, we also evaluated the antibacterial activity of RL (from 0.01 to 1%) against four individual bacterial strains: *B. megaterium* (KF951539), *Bacterium IMBL3* (HQ419191), *E. mundtii* (HQ419189), and *B. cereus* (JN559873). The MIC value for 0.01% RL was very low against the four bacterial strains (data not shown). Agar well diffusion assay showed inhibition at 0.25 with a maximum at 0.5 and 1% RL (figure 3).

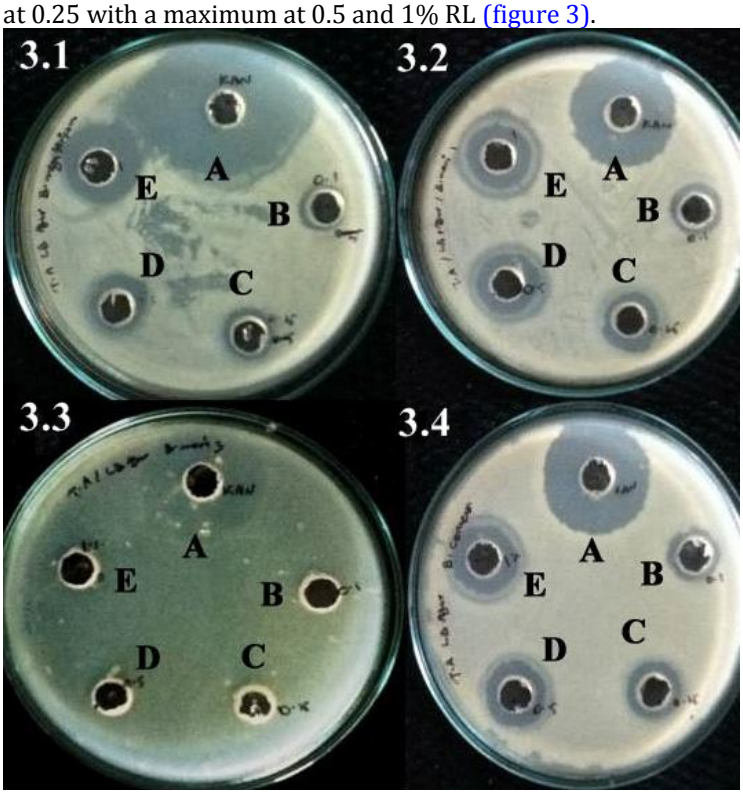

Figure 3: Agar well diffusion assay for Minimal Inhibitory Concentration of rhamnolipid. *Bacillus megaterium* (3.1) *E. mundtii* (3.2), *Bacterium IMBL1* (3.3) *Bacillus cerus* (3.4). A: Kanamycin (50ug/mL), B: 0.1% RL, C: 0.25% RL, D: 0.5% RL, E: 1% RL.

The graphical representation of MIC for antibacterial activity of RL is shown in figure 4.

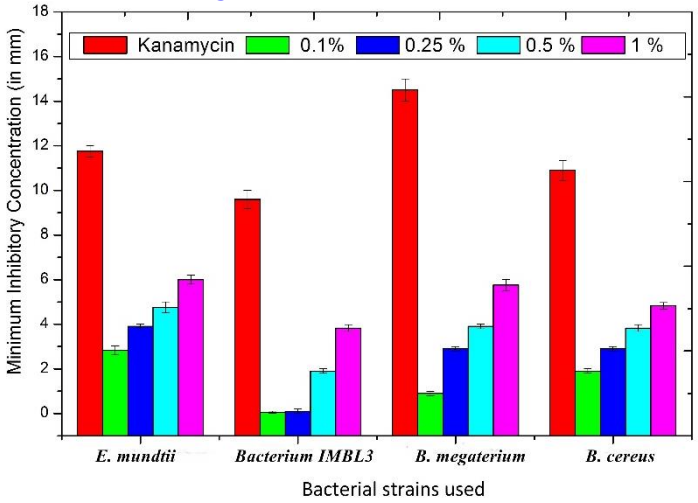

Figure 4: Graphical representation for Minimal Inhibitory Concentrations of rhamnolipid on gut bacteria. Values show the mean percentage and standard error of MIC.

Kanamycin (50µg/mL) was used as a control and showed complete inhibition of growth with 14 mm of zone formation. The higher MIC value for 0.5 and 1% RL demonstrates the susceptibility of bacterial species of *B. mori* gut towards RL. Eventhough antifeedant activity of RL was reported by Kama *et al.* (2012), the present study revealed that *invivo* antimicrobial activity of RL against larval gut microbes may be caused the insecticidal activity.

Besides microbes, gut proteins play a major role in digestion, innate immune response and tissue remodeling during larval to pupal transition (Kannan *et al.*, 2016). Therefore, it would be interesting to evaluate the potential effects of RL on gut proteomics. Summarizing, the results of this study clearly prove that the depletion of the host beneficial microbes in the larval gut, due to the *in vivo* antimicrobial properties of rhamnolipid, leads to indigestion and causes toxicity in *B.mori*. Further works are required to formulate RL-based biopesticides.

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CONFLICT OF INTEREST: On behalf of all authors, the corresponding author states that there is no conflict of interest.

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