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Gut microbial diversity and immunological effects of antibiotics on Spodoptera littoralis feeding on different diets

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Abstract

Insect gut microbes played important roles in host feeding, digestion, immunity, growth, and development. The use of antibiotics to remove or inhibit gut bacteria is a commonly used method to study the bacterial gut function of insects. In the present study, the effects of different concentrations of antibiotic mixture on the biological aspects, diversity of the midgut bacteria, and immunity of *S. litoralis* larvae were examined. The larvae were reared on three different diets. The results indicated that administration of the antibiotic mixture at concentrations ranging from 62 to 500 µg/mL significantly increased the survival rate within the three diets. While the pupal eight was significantly decreased in all diets. The number of midgut bacteria was significantly reduced with the increase of antibiotic concentrations. The gut bacterial diversity of *S. litoralis* larvae was surveyed and comprised of Bacilli and Gammaproteobacteria on larvae reared on the three diets. While Betaproteobacteria restricted only within the midgut of larvae reared on castor bean leaves. Also, when larvae reared on a diet amended with 500 µg/mL of antibiotic mixture, the midgut bacteria disappeared, and a reduction of cellular immunity occurred. Finally, the results confirm that the use of antibiotics could develop control strategies to get rid of harmful insects without damaging of environment or beneficial organisms by removing the midgut bacteria of pest that are responsible for increasing the immune system.

Keywords: Antibiotics, *Spodoptera littoralis*, midgut bacteria, diet- immunity

1. Introduction

The cotton leaf worm, *Spodoptera littoralis* (Boisduval) (Lepidoptera, Noctuidae) is the most damaging pest of cruciferous, decorative ornamental plants, and herbs (**Lanzoni *et al.*, 2012**). Novel biorational strategies are needed to manage the pest population. Knowledge about gut microbial symbionts associated with insects is a key step to develop novel control strategies (**Gowthami *et al.*, 2019**). Microbial symbionts such as bacteria, fungus, and viruses are abundant in the gut of insects and are responsible for insect fitness, survival, and immunity (**Jing *et al.*, 2014**). Numerous factors can influence the interactions between microorganisms and their gut host including type of diet, gut pH, digestive enzymes, age of insect, environment, physiological condition, and antibiotics (**Chandler *et al.*, 2011; Priya *et al.* 2012; Parian and Limketkai 2016**). Previous studies determined the abundance of bacterial communities in the gut of lepidopteran larvae according to nutrition type (**Xia *et al.*,2020; Mason *et al.*,2021; Lv *et al.*,2021**). Reducing or removing symbiotic gut bacteria by antibiotics is an essential step in studying the function of gut bacteria (**Dickel *et al.*, 2016; Xia *et al.*, 2018; Galarza *et al.*, 2021).** However, when individuals were treated with antibiotics, their survival rate, growth rate, and fitness were significantly reduced **(Gowthami *et al.*, 2019; Gao *et al.*, 2020; Van den Bergh, 2022).** Also, antibiotic treatment can increase insect immunity (**Xia *et al.*, 2018**). For example, phenoloxidase enzyme activity (Po) in wood tiger moth larvae reared on fumagillin antibiotic was higher than in normal larvae (**Dickel *et al.*, 2016**). **Rafiq *et al.* (2020)** used three types of antibiotics (ceftiofur sodium, oxytetracycline and enroflaxcin) to investigate their effects on cellular immunity of the silkworm, *Bombyx mori*. The result indicated that total haemocyte count (THC) in all larvae reared on diets that were amended with antibiotics was significantly increased compared to the control larvae. The current study aimed to determine the influence of antibiotics on biological and immunological aspects as well as the midgut bacteria diversity of *S. littoralis* larvae, which were reared on three different diets.

2. Material and methods

Insect rearing conditions

The colony of *S. littoralis* was obtained from the Cotton Pest Research Department, Agriculture Research Center, Egypt, and reared in the laboratory under constant conditions of temperature and humidity (26±4 °C and 65±5% RH). *S. littoralis* larvae were divided into three groups based on the rearing diets. The first group was reared on castor bean leaves (*Ricinus communis*), the second on clover leaves (*Trifolium alexandrinum*), and the third group on an artificial diet. The recipe of the artificial diet was 180 g yellow lentils, 25 g rice, 18.5 g brewer's yeast powder, 3 g ascorbic acid, 4 g sorbic acid, 2.5 g sodium benzoate, 1 mL formalin (40%), and 575 mL tap water. Lentils and rice were cooked in water for 20 min. After cooling, ascorbic acid, sorbic acid, and yeast powder were added to the mixture. The whole mix was blended in an electric blender, and then formalin was added to the blended homogenous mix **(Alfazairy *et al.*, 2012)**.

Antibiotic preparation

Four concentrations (62,125, 250, and 500 µg of antibiotic powder/100 mL of distilled water) were prepared from a mixture of three antibiotics (Cefoxitin,

Gentamycin, and Vancomycin) in a ratio of 1:1:1 according to **Esmael *et al.*** (**2022**). Leaf discs of fresh castor bean (each about 3.5 cm wide × 6.5 cm length) and Egyptian clover leaves (about 6 leaves to equal a caster leave disc) were pricked with a needle, washed with distilled water and 70% alcohol for 2 min., then dipped into serial dilutions of antibiotic solution for 20 min. and dried in an air vacuum within a laminar hood to ensure purity. About 20 gm of artificial diet (to equal leaf discs) was mixed well with mixed antibiotic powder. The control group larvae were fed the normal diet without antibiotic mixture.

Effect antibiotics on the development of *S. littoralis*

All egg patches (except the control group) were sterilized according to **Broderick *et al.*, 2009.** To avoid contamination, each egg patch was rinsed three times with sterilized solution (a mixture of Tween-80, bleach, and distilled water) for three seconds and dried under a vacuum hood before being placed in rearing containers and kept in an incubator (26±4 °C and 65±5% RH). 20 newly hatched larvae were placed in a container and reared on each corresponding diet, amended without or with the antibiotic cocktail (as mentioned earlier). The antibiotic-treated diets were replaced every day till the larvae pupated. Newly emerged pupae were transferred into glass jars containing sawdust until adult emergence. The newly adults were fed on a 10% sugar solution, and the jars were covered with a cotton cloth for egg laying. Total life cycle duration and weight of pupae were recorded. Four replicates were used for each concentration.

The midgut bacterial diversity of 5th*S. littoralis* larvae

Five 5th larval instars at one day-old (representing each diet and each antibiotic concentration) were starved for 24 h. Before dissection, larvae were washed by dipping into 70% ethanol for 2 seconds, followed by rinsing three times with distilled water. The midguts were dissected and crushed with 1 mL of phosphate-buffered saline (PBS) under aseptic conditions in the biological cabinet. The crushed midguts were diluted with sterile double distilled water to isolate midgut bacteria. 10 µL of each diluent was spread on each nutrient agar medium for 48 h under normal growth conditions (37±1°C). The number and type of bacteria in each midgut extract were calculated and represented as colony-forming unit/mL (CFU/mL). The midgut isolated bacteria were identified by VITEK® 2 COMPACT automated instrument for ID/ AST testing (Animal Health Research Institute, Cairo, Egypt) (**Pincus, 2006**) and Partial 16S rRNA gene sequencing was generated at Applied Biotechnology Company (Ismailia, Egypt) (**Kumar *et al.*, 2018**). The experiment was repeated four times for each diet and concentration.

The cellular immunity of 5th*S. littoralis* larvae

To investigate the possible effect of the antibiotic rearing on the immunity of 5th instar larvae of *S. littoralis*, fifty larvae (control and free-bacterial larvae that reared on diet amended with 500 µg/mL of antibiotic mixture) from each diet were starved for 24 h to assess THC and Po activity that represent the cellular immunity. The experiment was repeated four times.

After 24 and 48 h, the hemocytes were counted in the hemocytometer chamber according to **Manogem *et al.* (2015)**. In addition, Po activity was assayed according to **Mirhaghparast *et al.* (2013)**. The collected hemolymph from larvae was mixed with anticoagulant buffer and centrifuged at 13,000 rpm for 5 min.; the supernatant was discarded, and the pellet was washed gently twice with a phosphate buffer (0.02 M, pH = 7.1). Cells were homogenized in 200 µL of phosphate buffer, and 200 µl of catechol solution (2%) centrifuged at 13,000 rpm for 15 min. Samples (10 µl) were incubated with phosphate buffer at 25 °C for 3 min before the addition of 20 µL of 10 mM aqueous solution of L-dihydroxyphenyl alanine as substrate. The mixture was incubated for 5 min at 30 °C and the PO activity was determined as optical density units (O.D.) at an absorbency of 405 nm.

Statistical analysis

All data were presented as means ± standard error (SE) from four replicates. The significance of the differences in biological parameters and the quantity of midgut bacteria between the control groups and antibiotic treatment groups was determined using One-way analysis of variance (ANOVA, SPSS version 20.0). The significance level was set at a value of $P \leq 0.05$

3. Result

The influence of mixed antibiotics on the biological aspects of S. littoralis

Table (1) illustrated the influence of different concentrations of mixed antibiotics (Cefoxitin, Gentamycin, and Vancomycin) on S. littoralis development. With increasing in antibiotic concentration, the total life cycle was increased Fig (1). At 500µg/mL of antibiotic mixture, the survival rate was significantly increased in all diets. Regarding the pupal weight Fig (2), the untreated pupae had significantly the heaviest weight (257mg) when larvae were reared on an artificial diet followed by castor bean (248mg) , and clover leaves (224.5mg). But in case of antibiotic treatment, the pupal weight decreased with the increasing antibiotic concentration in all diets ($P \leq 0.05$).

Table (1) The influence of mixed antibiotics on the biological aspects of *S. littoralis* larvae reared on different diets

Conc. of mixed antibiotics• (µg/mL)	Biological parameters of *S. littoralis* reared on different diets (Mean ± SE)					
	Castor beans		Clover		Artificial diet	
	Life cycle duration/day	Pupal weight/mg	Life cycle duration/day	Pupal weight/mg	Life cycle duration/day	Pupal weight/mg
Control (0)	44.0± 1.6^{c}	248.00±4.60^{a}	57.00± 1.73^{c}	224.50±3.52^{a}	52.00 ±2.04^{b}	257.50±0.96^{a}
62	54.00±1.78^{b}	231.00±3.49^{b}	67.75±2.25^{b}	219.00±6.38^{a}	58.00±1.41^{b}	251.00±0.82^{a}
125	60.25±2.27^{ab}	216.75±3.95^{c}	73.0±1.32^{a}	211.25±7.67^{a}	62±0.85^{ab}	243.00±1.47^{a}
250	67.5±2.25^{a}	197.50±7.92^{d}	75±1.08^{a}	187.75±0.95^{b}	69±1.58^{a}	221.75±1.44^{b}
500	69.5±1.56^{a}	194.50±2.06^{d}	79.25± 1.25^{a}	181.25±2.36^{b}	76.25. ±0.63^{a}	207.00±3.08^{c}

a, b, c&d: There is a significant difference (P≤0.05) between any two means, within the same Colum have the same superscript letter.

•: mixed antibiotics (Cefoxitin, Gentamycin, and Vancomycin) in a ratio of 1:1:1

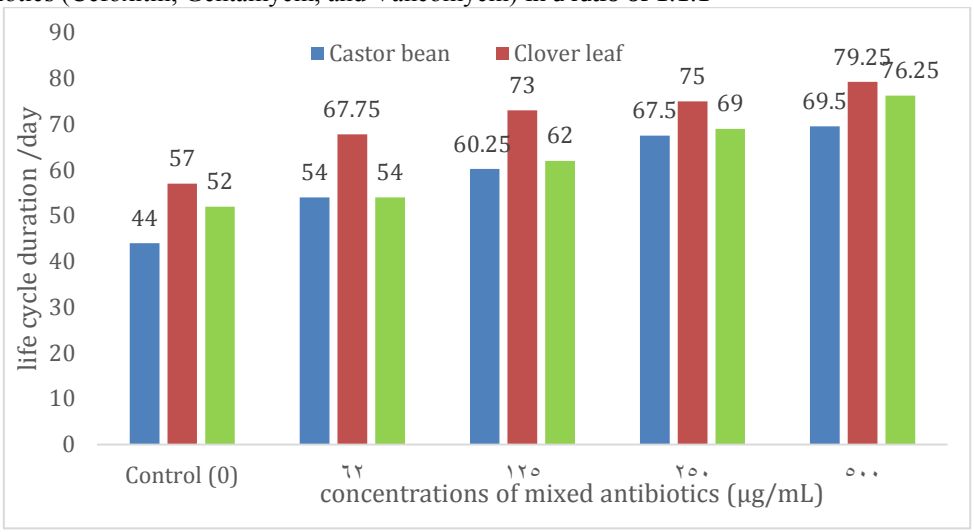

Fig. (1) Effects of mixed antibiotics on the life cycle duration of *S. littorals* larvae reared on different diets.

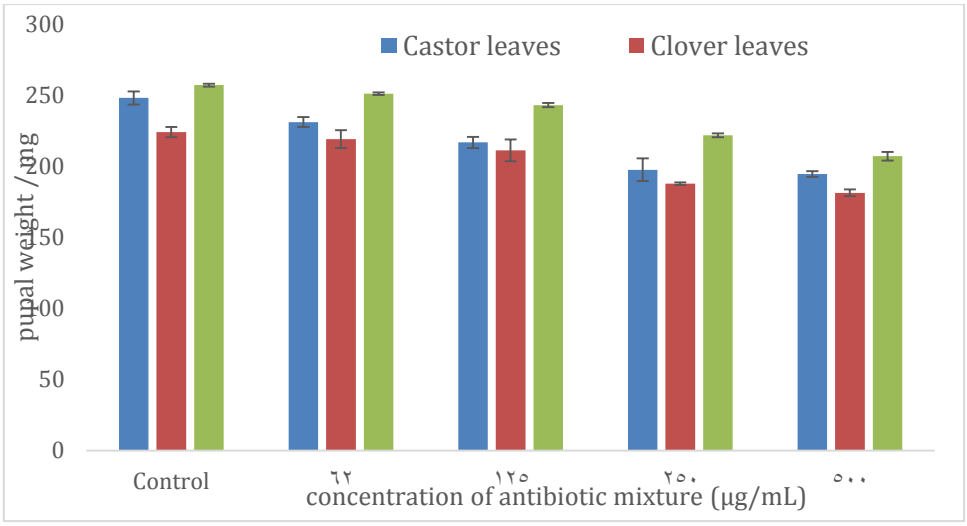

Fig. (2) Effects of mixed antibiotics on the pupal weight of *S. littorals* according to different diets.

The abundance of midgut bacteria

In case of control, the number of the midgut bacteria of the artificial diet was significantly reduced ($7.7*10^{7}$ CFU) compared with other diets that recorded $11.85*10^{7}$ and $19*10^{7}$ CFU for clover and castor bean, respectively. Based on the results (Table 2, Fig. 3), the abundance of midgut bacteria in the control group was the highest and very steady. While the larvae fed on antibiotics, the midgut bacteria were significantly reduced with increasing antibiotic concentrations. The midgut bacteria could not be detected on the media at mixed antibiotic concentrations of 500 µg/mL.

Table (2) Numbers of bacterial midgut (CFU) of 5th instar *S. littoralis* larvae reared on mixed antibiotics (Cefoxitin, Gentamycin, Vancomycin) on different diets.

Type of diet	CFU*10^7 Concentration of antibiotic mixture (µg/mL)				
	Control	62	**125**	**250**	**500**
Castor	19±0.71^a	18.8±0.21^a	14.75±0.25^b	7.75±0.63^c	0±0^d
Clover	11.85±0.38^a	11.95±0.58^a	9±0.08^b	2.775±0.08^c	0±0^d
Artificial diet	7.7 ±0.14^a	8.6±0.27^a	6.575±0.24^a	2.425±0.11^b	0±0^c

a, b, c &d: There is significant difference (P≤0.05) between any two means, within the same raw have the same superscript letter.

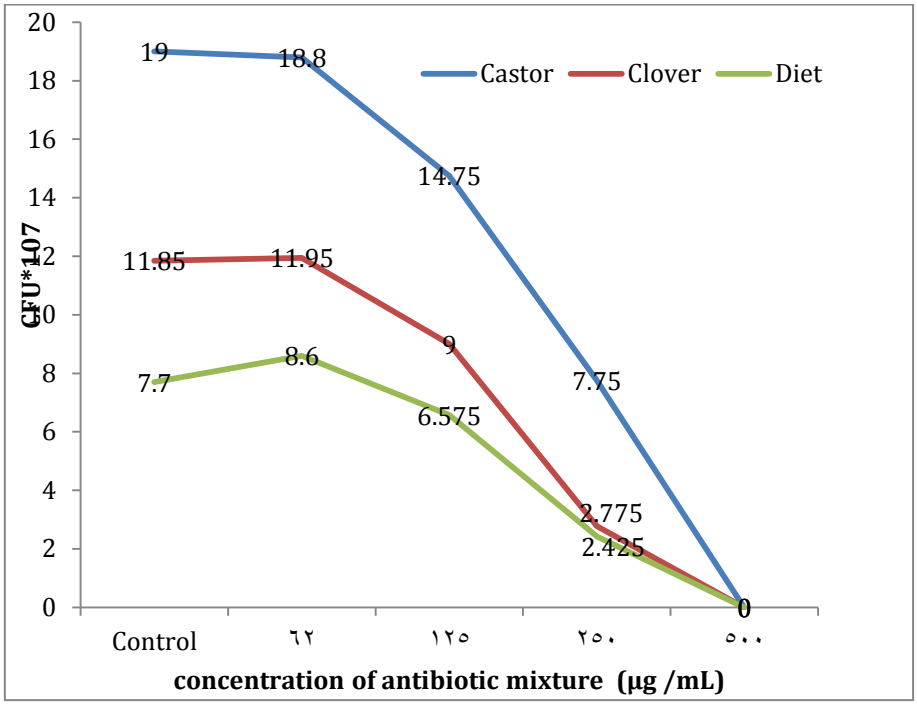

Fig. (3) CFU of 5th instar *S. littoralis* larvae reared on mixed antibiotics (Cefoxitin, Gentamycin, Vancomycin) on different diets.

Diversity of midgut bacteria in 5th larval instar of *S. littoralis*

In control group, the isolated bacteria were categorized into a total of 2 phyla (Firmicutes and Proteobacteria), 11 genera, and 13 species (Table 3). Staphylococcus gallinarum, Enterococcus casseliflavus, Buttiauxella agrestis, and Ralstonia pickettii were isolated from midguts of larvae reared on castor bean leaves; Klebsiella oxytoca, E. casseliflavus, Enterococcus gallinarum, and Mammaliicoccus sciuri from the midgut of clover feeding larvae, while the bacterial midgut of larvae feeding artificial diet were Paenibacillus polymyxa, Lysinibacillus fusiformis, M. sciuri, Serratia plymuthica, and Lactobacillus acidophilus (Table 3). All species of culturable bacteria isolated from 5th instar larvae of S. littoralis that reared on three different diets amended with antibiotic mixture were significantly decreased (Table 4). At 250µg/mL of antibiotic treatment, only six bacterial strains (B. agrestis, E. casseliflavus, K. oxytoca, M. sciuri, S. plymuthica and L. acidophilus) were cultured successfully on the media. Among three diets, the 500 µg/mL concentration of antibiotic mixed removed all bacterial isolated from midgut of 5th larval instar.

Table (3) Enteric bacteria in the midgut of the 5th larval instar of *S. littoralis* identified by the VITEK automated system and 16S rRNA technique according to the diet

Diet	Bacterial isolates	Family	Class	Phylum
Castor bean leaf	Staphylococcus gallinarum	Staphylococcaceae	Bacilli	Firmicutes
	Enterococcus casseliflavus	Streptococcaceae		
	Buttiauxella agrestis	Enterobacteriaceae	Gammaproteobacteria	Proteobacteria
	Ralstonia pickettii	Burkholderiales	Betaproteobacteria	
	Klebsiella oxytoca	Enterobacteriaceae	Gammaproteobacteria	Proteobacteria
Clover leaf	Enterococcus gallinarum	Streptococcaceae	Bacilli	Firmicutes
	Enterococcus casseliflavus	Enterococcaceae		
Artificial diet	Mammaliicoccus sciuri	Staphylococcaceae	Bacilli	Firmicutes
	Lysinibacillus fusiformis	Bacillaceae		
	Mammaliicoccus sciuri	Staphylococcaceae		
	Lactobacillus acidophillus	Lactobacillaceae		
	Paenibacillus polymyxa	Paenibacillus		
	Serratia plymuthica	Yersiniaceae	Gammaproteobacteria	Proteobacteria

Table (4) the effect of mixed antibiotics on numbers of midgut bacteria isolates according to three different diets after 48h of treatment.

type of diets	Bacterial isolates	CFU *107 Concentration of antibiotic mixture (µg/mL)				
		Untreated Control	62	125	250	500
Castor bean leaf	**Staphylococcus gallinarum**	4.75±0.48^{a}	4.25±0.25^{a}	3±0^{a}	0±0^{b}	0±0^{b}
	Buttiauxell aagrestis	9.00±0.58^{a}	8.5±0.5^{a}	6.75±0.48^{ab}	4±0.41^{b}	0±0^{c}
	Ralstonia pickettii	36.25±1.70^{a}	16.25±1.7^{b}	0±0^{c}	0±0^{c}	0±0^{c}
	Enterococcus casseliflavus	7.00±0.41^{a}	6.5±0.41^{a}	5±0.58^{a}	3.75±2.23^{a}	0±0^{b}
Clover leaf	**Klebsilla oxytoca**	41.50±1.44^{a}	40.5±2.1^{a}	36.25±0.75^{a}	20.25±1.25^{b}	0±0^{c}
	Enterococcus gallinarum	48.25±3.35^{a}	44±3.79^{a}	39.5±0.96^{a}	25±1.23^{b}	0±0^{c}
	Mammaliicoccus sciuri	17.50±1.85^{a}	15.25±1.44^{a}	9±1.08^{b}	0±0^{c}	0±0^{c}
	Enterococcus casseliflavus	11.25±1.11^{a}	10.25±0.63^{a}	5.25±0.85^{b}	2.75±0.48^{b}	0±0^{c}
Artificial Diet	**Lysinibacillus fusiformis**	14.25±1.80^{a}	13. 5±1.26^{a}	9±1.47^{b}	0±0^{c}	0±0^{c}
	Paenibacillus polymyxa	9.25±0.85^{a}	8±0.63^{a}	7.25±0.85^{a}	0±0^{b}	0±0^{b}
	Serratia plymuthica	30.75±1.25^{a}	22±1.47^{ab}	19±1.83^{b}	9.5±0.96^{c}	0±0^{d}
	Mammaliicoccus sciuri	22.75±1.32^{a}	18±5.43^{ab}	15.5±1.32^{ab}	7±0.82^{b}	0±0^{c}
	Lactobacillus acidophillus	21.75±1.38^{a}	19.25±1.11^{a}	15±2.04^{ab}	7.75±0.48^{b}	0±0^{c}

a, b, c& d: There is significant difference (P≤0.05) between any two means, within the same row have the same superscript

Cellular immunity

Based on control result (Fig. 4), the highest THC (10825 mm/ cm^{3}) and Po activity (13.8 OD/min.) values were observed in haemolymph collected from 5th instar larvae reared on clover diet, while castor bean leaves recorded the lowest value of THC (8287.5 mm/cm^{3}) and Po (5.9 OD/min.) activity. In all three diets, after larvae reared on diet amended with 500 µg /mL of antibiotic mixture, both THC and Po activity increased after 24 and 48h of treatment in 5th instar larvae than the control.

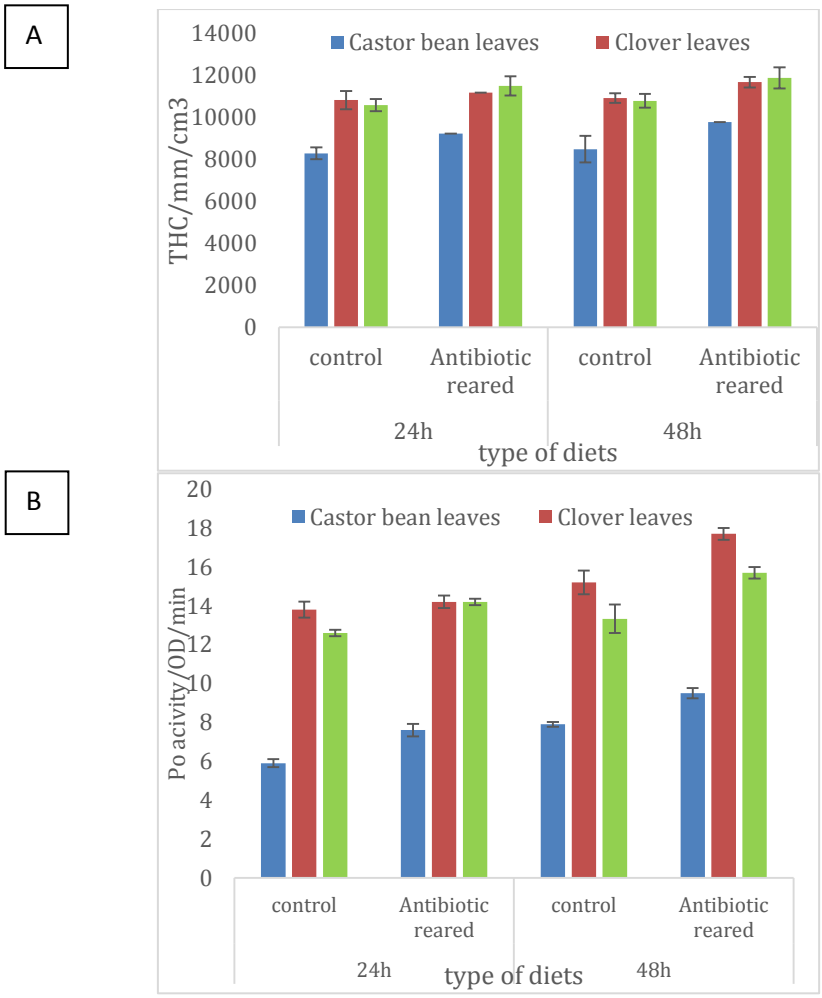

Fig. (4) The influence of antibiotic on immunity of 5th instar *S. littoralis* larvae were reared on three different diets for 24 and 48 h. **A**: THC, **B**: Po.

4.Discussion

The results revealed that the lifecycle duration of *S. littoralis* larvae was significantly the shortest on castor bean leave diets, while the longest lifecycle was detected n clover leaves. The same results were obtained by **El–Aw and Hashem (2004)**; **Shekhawat *et al.* (2018)**; **Mohamed *et al.* (2019)**. On the other hand, **Matar *et al.* (2012)** reported the longest lifecycle of S. *littoralis* on castor bean leaves. This might be due to castor compositions have a high amount of carbohydrates, total nitrogen, proteins, and microelements that are essential for the skeleton build-up process of the early instars and the shortest larval period (**Sreelakshmi and Mathew 2017; Ismail, 2020**).

After larvae reared on a range of concentrations (62–500 µg/mL) of an antibiotic mixture that consisted of Cefoxitin, Gentamycin, and Vancomycin, the life cycle period was significantly increased in all three diets. The results are consistent with the previous reports of **Goharrostami and Sendi (2018)** who observed prolonged development period in *Ephestia kuehniella* larvae reared on diet amended with streptomycin sulfate and tetracycline antibiotic. On the other hand, **Yang *et al.* (2020)** showed that survival time of *Plutella xylostella* larvae reared on antibiotic treated diet had significantly lower period than the control larvae. Also, **Dickel *et al*. (2016)** examined the effects of prophylactic antibiotic treatment on the survival and life-history traits of *Parasemia plantaginis*. The authors indicated that the prophylactic antibiotic decreased the insect developmental time.

Regarding to the pupal weight, it was significantly decreased with the increase of antibiotic concentration in all diets. Similarly, **Wang *et al.* (2014)** recorded reduction of pupal weight of *Cnaphalocrocis medinalis* after rearing on artificial diet treated with three antibiotics (Erythromycin, Chloramphenicol, and Spectinomycin). The reduction of pupal weight occurred might be due to the direct effect of antibiotics on the gut cells of the larvae and its negative effect on normal growth and metabolism (**Lin *et al.*, 2015).** Also, the indirect effect of antibiotics was killed most of the gut bacteria, including beneficial microorganisms, and created gut flora disorder (**Xia *et al.*, 2018**).

Insect diet is the basic factor that greatly influences insect gut microbiota (**Jones *et al.*, 2018;**

Mason *et al.*, 2021). In the current study, the highest bacterial frequencies were isolated from insects reared on castor bean leaves followed by insects reared on clover leaves than in the artificial diet. This is probably because of the higher protein content present in the castor bean leaves which favors the development of bacteria (**Ismail, 2020**). **Visôtto *et al.* (2009)** noted the variability in gut bacterial counts of lepidopteran larvae according to the diet. As well as **Yang *et al.* (2020)** observed a reduction in the quantity of bacterial gut of *P. xylostella* larvae reared on radishes than that reared on peas leaves. But, the number of bacterial midgut of *S. littoralis* larvae reared on three diets amended with mixed antibiotics could be significantly reduced. Similarly, **Gowthami *et al.* (2019)** investigated the effects of eight antibiotic mixed (Amoxicillin, Cephalexin, Doxycycline, Cefixime, Cefpodoxime, Ciprofloxacin, Levofloxacin, and Tetracycline) on the gut bacterial quantality of *P. xylostella*. Due to antibiotic exposure, gut bacterial number was significantly reduced. Also**, Gao *et al.* (2020)** evaluated different concentrations of mixed antibiotics on gut bacteria of *Ectropis obliqua*. The results indicated that the administration of high concentration of mixture antibiotics removed all gut bacteria

The isolated bacteria were categorized into a total of 2 phyla (Firmicutes and Proteobacteria), the data showed a higher abundance percentage of the Proteobacteria than the Firmicutes when *S. littoralis* insects were reared on either the castor or clover leaves. However, when *S. littoralis* insects were constantly reared on artificial diets the insects harbored higher bacterial communities in the Firmicutes phyla than in the Proteobacteria. **Xia *et al.* (2020)** reported that Proteobacteria and Firmicutes were the dominant bacterial phyla in the gut of insects especially, in Lepidopteran species. This finding might be due to their functional roles in carbohydrate metabolism, amino acid metabolism, and membrane transport pathways of the host (**Wang *et al.* 2020**).

Furthermore, insects reared on the castor bean leaves harbored four isolates. For the insects reared on the artificial diet, five isolates were detected. Also, these bacterial isolates significantly reduced after larvae reared on antibiotics, while at the concentrations of 500µg/mL of mixed antibiotics; all bacteria isolates completely removed. Similarly, **Van den Bergh (2022)** treated *Drosophila melanogaster* larvae with different concentrations of antibiotics then observed decreased in bacterial microbiota inside larval gut. **Kohanski *et al.*,2010; Dowling *et al.*, 2017** indicated that the changing or removing of bacterial gut within larvae reared on antibiotic might be due to its effect as inhibitor to the bacterial cell wall synthesis (Beta-lactam antibiotics), nucleic acid synthesis (Fluoroquinolone), or ribosome function (Tetracycline).

Also, this study clarified the influence of antibiotic on the immunity of insect. The THC and PO activity of 5th larval instar of *S. littoralis* reared on three different diets amended with 500 µg/mL of mixed antibiotic was more increased than normal larvae. This result agreed with **Dickel *et al.*, 2016** who observed increasing in Po activity of larvae of wood tiger moth after rearing on diet amended with fumagillin antibiotic. As well as, the THC of third larval instar reared on mulberry leaves amended with mixed antibiotics (ceftiofur sodium, oxytetracycline and enroflaxcin) was significantly increased than control larvae **(Rafiq *et al.*, 2020)**. In contract, **Galarza *et al.* (2021)** found decrease in immunity of wood tiger moth after treatement with two broad-spectrum antibiotics, tetracycline, and ciprofloxacin. Also**,** both **Duan *et al.*, 2021; Fu *et al.*, 2021** observed a decrease in immunity of Honeybee larvae treated with penicillin–streptomycin antibiotic

Conclusion

The present investigation indicated that antibiotic influenced on biological developmental parameters and the pupal weight of *S. littoralis.* These might be the negative effect of antibiotics on the normal growth and metabolism. Also, the antibiotics showed the ability to remove the midgut bacteria due to antibiotics acting as inhibitor to the bacterial cell wall synthesis, nucleic acid synthesis, or ribosome. In addition, the larvae reared on antibiotic had THC and Po activity more than the control due to the removing of bacteria that active the immune system.

Conflicts of interest

There is no conflict of interest.

Acknowledgment

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