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ORIGINAL RESEARCH ARTICLE

Midgut health of *Apis mellifera* workers on oral administration of *Lactobacillus rhamnosus* and organic acids

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ABSTRACT

A study about the provision of *Lactobacillus rhamnosus* NR_113332) and organic acids was carried out. 10^8 colony-forming units of probiotics were provided to the caged workers with and without the addition of 2.99% lactic acid, 2.91% acetic acid, and 1.96% acetic acid. After two weeks of oral administration, on termination, five bees were collected and dissected from each cage, and their midguts were cut into 8 µm thick sections by microtome. Subsequently, these histologically processed preparations were observed under camera fitted microscope. It was observed that the experimental workers were provided with diets: (pollen+ 50% w/v sucrose in 1.96% acetic acid, pollen+ *L. rhamnosus* in 50% w/v sucrose in distilled water, pollen+ *L. rhamnosus* in 50% w/v sucrose in 2.99% lactic acid, pollen+ *L. rhamnosus* in 50% w/v sucrose in 2.91% acetic acid, pollen+ *L. rhamnosus* in 50% w/v sucrose in 1.96% acetic acid) had a considerable quantity of peritrophic membranes. The mean midgut lumen diameters (µm^2) were 373.33 ± 98.38, 296.67 ± 23.33, 243.33 ± 71.72, 426.67 ± 40.55, 576.67 ± 93.87 as compared to control's 240.00 ± 30.55. The provision of probiotic and organic acids resulted in a slightly disturbed midgut epithelial wall, the presence of more quantity of peritrophic membranes, saved regeneration crypts, more gastric cells discharging, and some cytoplasmic vacuolization of caged worker bees. Our study suggests that probiotics and organic acids can be used as an environmentally friendly apipromotor, but verification by field studies is needed.

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Probiotics; organic acids; midgut; *Apis mellifera*; Histological changes

Introduction

Honeybees (*Apis mellifera*) are important pollinators that have a great impact on agriculture worldwide (Le Conte & Navajas, 2008) and they are also well-known for the monitoring of environmental pollutants (Celli & Maccagnani, 2003). The digestive system is a key organ for honey bee health as it is the site of contact with pathogens and xenobiotics (Han et al., 2012). The digestive system of insects consists of three parts: the foregut, midgut, and hindgut. In honey bees, the foregut is composed of the pharynx and esophagus, the midgut is composed of the ventricle/true stomach, and the hindgut is composed of the ileum and rectum (Santos & Serrão, 2006). The ventricle is considered to be the main organ for pollen digestion and absorption, while the ileum and rectum are considered to be mainly responsible for controlling osmotic pressure by absorbing water and ions (Gajger et al., 2011; Szymas et al., 2012). The midgut and intestine are the main organs affected by negative stimulants. The midgut epithelium is responsible for the detoxification of ingested xenobiotics (Higes et al., 2013). It is the only tissue in adult bees that exhibits extensive cell proliferation (Ward et al., 2008). Therefore, an important organ for toxicity analysis is the midgut, as it is responsible for the digestion and absorption of ingested food. The midgut and intestinal mucosa act as a protective shield that induces an immune response to many honey bee pathogens (Szymas & Przybył, 2007). For this reason, these organs are often the first areas to be attacked and infected by honey bee pathogens and other vectors (Das Dores Teixeira et al., 2015). The most abundant epithelial cell type is the columnar cells in the midgut, which are arranged in a layer and settle on the basement membrane, with regenerating cells in between (Cruz-Landim & Cavalcante, 2003; Serrao & Cruz-Landim, 1995). Probiotics alongwith prebiotics are being used to improve the overall health of honey bee's colony (Hasan et al., 2022Hasan et al., 2023b). To exploit the honey bees's potential, the concurrent use of pro and prebiotics (synbiotics) is gaining special attention these days. Acidifying

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agents or organic acids function like prebiotics and play their key role in lowering the gut pH and prohibit to grow the pathogenic microbes. This thing has experimentlly been proved that those bees which were fed on sugar syrup diet with pH between 3-6.5 had decreased number pathogenic microbes. The simultaneoususe of probiotics and acidifying agents exert beneficial impacts in such acway that organic acids prohibit the pathogen's growth and in the meantime the colonization of gut with beneficial microbes (probiotics) ultimately improves host's health and imunity (Patruica et al., 2013).

The present study was designed to observe the beneficial role of probiotics and selected organic acids on morphological/histological changes (existence of peritrophic membranes, cytoplasmic vacuolation, gastric cell discharging, impact on regeneration crypts) in midgut epithelium and mean lumen diameters of European bee (*Apis mellifera ligustica*) for fourteen days (two weeks).

Materials and methods

Experimental trials setup

According to Williams et al. (2013), honey bee combs were collected from the Punjab University honey bee research garden (31.49758° N; 074.29679 E) and were maintained in an incubator for the emergence of worker bees for experiments at 32 °C T and 65% RH. The internal conditions of the incubators were maintained after Williams et al. (2013). Zero to twenty-four (0-24) hours aged workers from *Apis mellifera ligustica* colony were selected for the experiments and put in plastic cages (11 cm × 6cm × 6cm) to be maintained in incubators at 32 °C temperature and 65% relative humidity. The current investigation was done employing plastic jar cages which were made following Hasan et al. (2022). Each cage was equipped with a feeder and a pollen provider, made with a 5 ml plastic syringe and a 2 ml capacity Eppendorf respectively. According to Evans et al. (2009) and Martín-Hernández et al. (2009), the cages were loaded with 50 freshly emerged bees and immediately transported to an incubator maintained at 3 °C and 70% humidity. Three replicates were considered for histological study in the current investigation. One bacterial probiotic (*Lactobacillus rhamnosus* NR_113332) was used in the current experiments along with two organic acids (lactic and acetic acid).

Sugar syrups preparation and provision

Following Patruica et al. (2013), the bees were provided with an acidifying agent by making stock solutions, i.e. 7.5 ml of acetic acid per 250 mL of dH2O, 5 ml of acetic acid per 250 mL of dH2O and 0.75 mL of lactic acid per 250 mL of dH20. To assess the effect of the organic acids, the experimental groups were provided with syrup mixed with sucrose and the respective acidifying agent in (1:2). Feeding details of caged workers fed on organic Acids and *Lactobacillus rhamnosus* with organic acids was same as descibed by Hasan et al. (2023b) as; Control: Pollen+ 50% w/v sucrose in DW pH: 8; Experiment 1 (Exp1-LA): Pollen+ 50% w/v sucrose in 2.99% LA pH: 3.14; Experiment 2 (Exp 2-AA(II): Pollen+ 50% w/v sucrose in 2.91% AA pH: 2.95; Experiment 3 (Exp 3-AA (I): Pollen+ 50% w/v sucrose in 1.96% AA pH: 3.12; Experiment 4 (Exp 4- Lr): Pollen+ *L. rhamnosus* in 50% w/v sucrose in DW pH: 8; Experiment 5(Exp 5-LaLr): Pollen+ *L. rhamnosus* in 50% w/v sucrose in 2.99% LA pH: 3.14; Experiment 6 (Exp 6-AA(II)Lr: Pollen+ *L. rhamnosus* in 50% w/v sucrose in 2.91% AA pH: 2.95; Experiment 7 (Exp 7-LrAA (I): Pollen+ *L. rhamnosus* in 50% w/v sucrose in 1.96% AA pH: 3.12. The pollen used as a dietary protein source was obtained from a honey bee research farm at the University of Punjab) and mixed in distilled water in a 9:1 (w/v) ratio to create a dough-like consistency for workers in cages following Alaux et al. (2010). The probiotic master stock was prepared following Hasan et al. (2022) and the dose of the prepared probiotic was maintained at 7 °C. Following Szymas et al. (2011) with slight modifications in the number of CFUs and carrier, each ml of syrup was provided to the caged bees with approximately 1 x 10^8 CFUs of probiotics mixed in sugar syrup. Each experimental cage received 4 ml of the syrup per day, Hence, the number of eppendorf tubes, (having 4 x 10^8 CFUs) were withdrawn from the stored master stock of probiotics and centrifuged at 15,000 rpm for 10 min at 25 °C. After discarding the supernatant, the pellets were soaked in their respective sugar syrups made on that day, vortexed, and presented to the caged bees. This procedure was repeated in all experimental groups where probiotic was provided to the bees.

Procedure of experimental termination

After two week's oral administration of probiotics and organic acids, three bees were randomly selected from each experimental group and placed in the freezer at −20 °C for 2 h to kill bees following Naug and Gibbs (2009). The tissues (midguts) of bees that were put to permanent sleep intentionally were then preserved in 10% formalin (AnalaR ® Product: 20909.330, Bach # 17A094006, VWR chemicals) till further histological analyses. Already dead bees in the cages were not used for the histological study to prevent the risk of tissue necrosis.

Dissection of honey bees

Bees were dissected using a dissecting binocular apparatus (ER-59-1828, Carolina biological supply company USA) after Carreck et al. (2013) and Hasan et al. (2022b). The items used in the dissection procedure included scissors, forceps, needles, a thick wire approximately 150 mm long, and Petri dishes filled with melted wax. The bees to be dissected were placed on blotting paper for a while before dissection so that the paper could absorb all the preserving material that had soaked the bees, and then the wings, legs, and proboscis were cut off with scissors for morphometric measurements. The resting bee was placed on wax, to expose its ventral side. Starting at the posterior end of the abdomen, two superficial incisions were made along the lateral side of sclerites that started at the posterior end of the abdomen and terminated close to the petiole. After the incisions, the gastrointestinal tract and other internal organs got incised and separated. The procedure was repeated for each experimental bee.

Histological studies

Histological changes in honey bee tissues of experimental groups were assessed after two weeks according to Bzymas et al. (2012). Bee tissues (e.g. midguts) fixed in 10% formalin were dehydrated in graded ethanol. The dehydrated tissues were cleared in xylene and then embedded in paraffin. Blocks were prepared using cavity glass and these blocks were cut into 8 μm thick sections using a rotary microtome (model MRS, Japan) and dried after mounting on glass slides. These slides were deparaffinized using xylene and then rehydrated in graded ethanol. Using Mayer's hematoxylin and eosin dyes, these sections were stained. Afterwards, these slides were once again dehydrated in alcohol, cleared in 3-4 passes of xylene, and then coverslipped. These preparations were examined under a high power microscope (Utech Schenectady, NY12305).

Statistical analysis

At the end of the experiment (after two weeks of administration of organic acids and probiotics), a one-way analysis of variance (ANOVA) was performed using SAS 9.1 to determine the difference between the mean midgut lumen diameters. Means were compared by applying Duncan's multiple range test (DMR) at a 95% confidence level.

Results

Bees of experiments No. 1 and 2 showed 100% mortalities and were hence excluded from the experiment. Control bees which were provided with pollen and 50% (w/v) sucrose in distilled water, exhibited the wall of their midguts wrinkled properly, the peritrophic membrane was in contact with rabdorium, nuclei were stained well, and columnar epithelial cells were arranged in good array. Gastric cells were noticeable. The mean lumen diameter was 240.00 ± 30.55 μm^2.

Worker bees that were fed on pollen and 50% (w/v) sucrose in 1.96% acetic acid had a slightly disturbed midgut wall, there were little spaces between some columnar epithelial cells, peritrophic membrane was clung with rabdorium mean lumen diameter was 373.33 ± 98.38 μm^2. Provision of pollen and *L. rhamnosus* in 50% (w/v) sucrose in distilled water resulted in epithelial cells being closer to each other, more numerous gastric cells discharging was observed, and pollen was wrapped in peritrophic membranes. Properly stained nuclei were seen mean lumen diameter was 296.67 ± 23.33 μm^2. Caged bees which were fed with pollen and *L. rhamnosus* in 50% (w/v) sucrose in 2.99% lactic acid depicted dented epithelial wall in some places, peritrophic membranes were somewhat attached to the columnar epithelial cells, gastric cells were more in number, mean lumen diameter was 243.33 ± 71.72 μm^2. On provision of pollen and *L. rhamnosus* in 50% (w/v) sucrose in2.91% acetic acid, worker bees had the stomach wall damaged and exhibited indentations, but regeneration crypts were saved, pollen was seen wrapped in peritrophic membranes and was closer to the epithelial wall, numerous gastric cells were also prominent, mean lumen diameter was 426.67 ± 40.55 μm^2. When pollen and *L. rhamnosus* in 50% (w/v) sucrose in 1.96% acetic acid were presented to the caged bees, they had the properly wrinkled midgut wall, the peritrophic membrane was in contact with rabdorium like control bee's midgut, and peritrophic membranes covered the rabdorium in whole midgut wall. Slight cytoplasmic vacuolization was seen. Epithelial cells were preserved better; the mean lumen diameter was 576.67 ± 93.87 μm^2, F and F1 (Plates 1 and 2, $p = 0.02$, $F = 3.75$, Table 1, Figure 1).

Discussion

The conclusions of our research elaborate on the influence of different treatments of probiotics, and organic acids on honey bee's digestive system specifically the epithelial wall of the midgut. It was found that our feed treatments did not influence the bee's stomach detrimentally. The provision of a mixture of protein substitute and probiotics to honey bees resulted in better consumption of feed which was proved by good condition, decreased death rates, development of fat body, pharyngeal glands, and increased concentration of dry matter of bees

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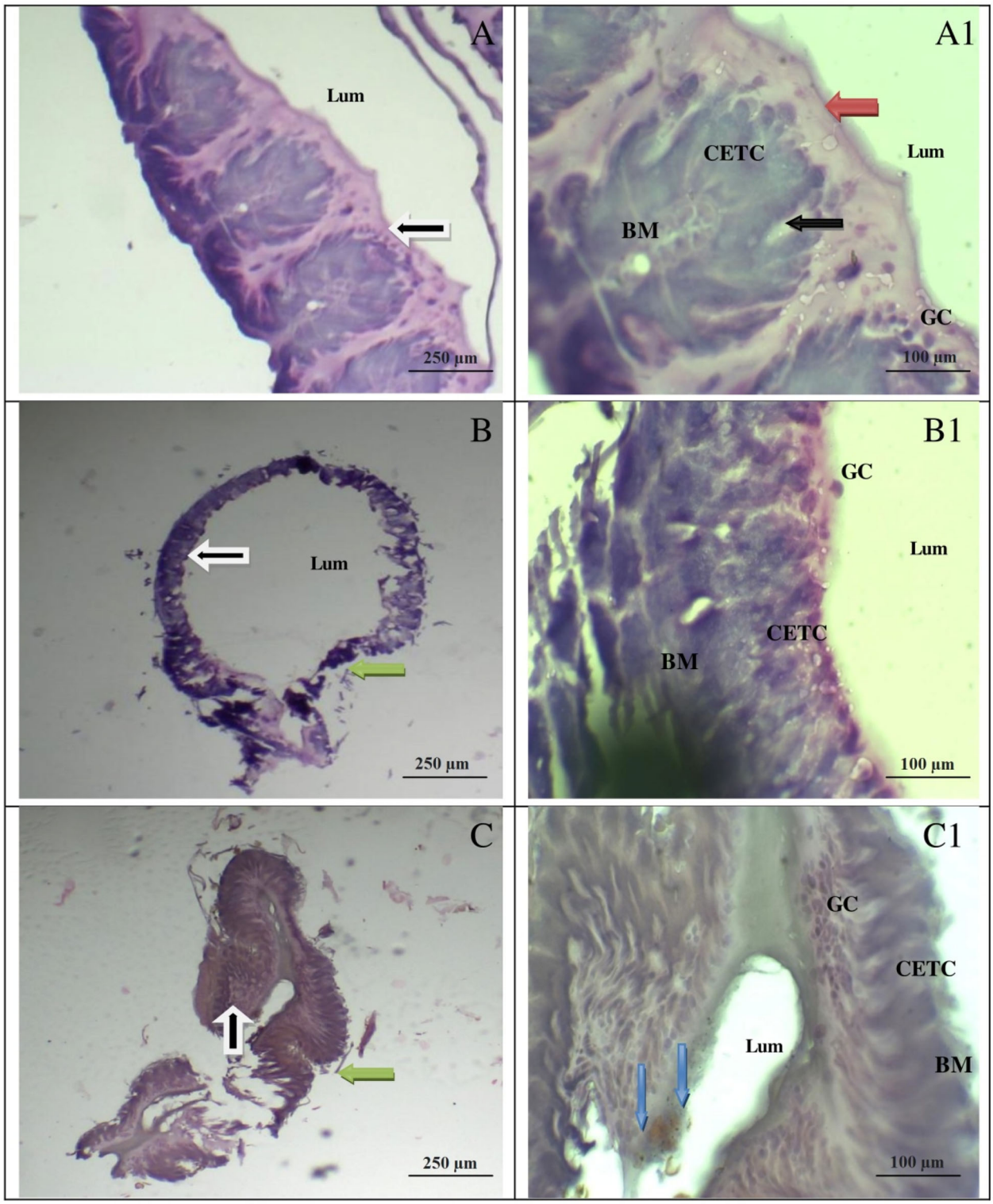

Plate 1. Control (A, A1), experiment 3 (B, B1), experiment 4 (C, C1)
Green arrow: muscle layer, Red arrow: peritrophic membranes, Blue arrow: pollen wrapped in peritrophic membranes, Black and white arrow: the highlighted portion in (A1, B1 and C1), A and C are LS of midgut while B and B1 are TS of midgut, Orange arrow: cytoplasmic vacuolization, BM: basement layer, CETC: columnar epithelial cells, Lum: lumen, GC: gastric cells, Dark grey arrow: regeneration crypts

(Kaznowski et al., 2005; Łangowska et al., 2003). In the current experimental groups, feeds doped with organic acids and probiotics were consumed more willingly by experimental workers and their deat rates were lesser than the control group.

The functional cells in the digestive tract are columnar epithelial cells whereas to redevelop the damaged functional cells, regenerative cells are also present (Cruz-Landim et al., 1996; Serrao & Cruz-Landim, 1995). In the current experimental study, gastric cells discharging were seen, and regeneration crypts remained saved. Midgut provides a basic platform for the absorption and digestion of chemicals and nutrients in insects. In most insects, food is wrapped in the cellular layer which

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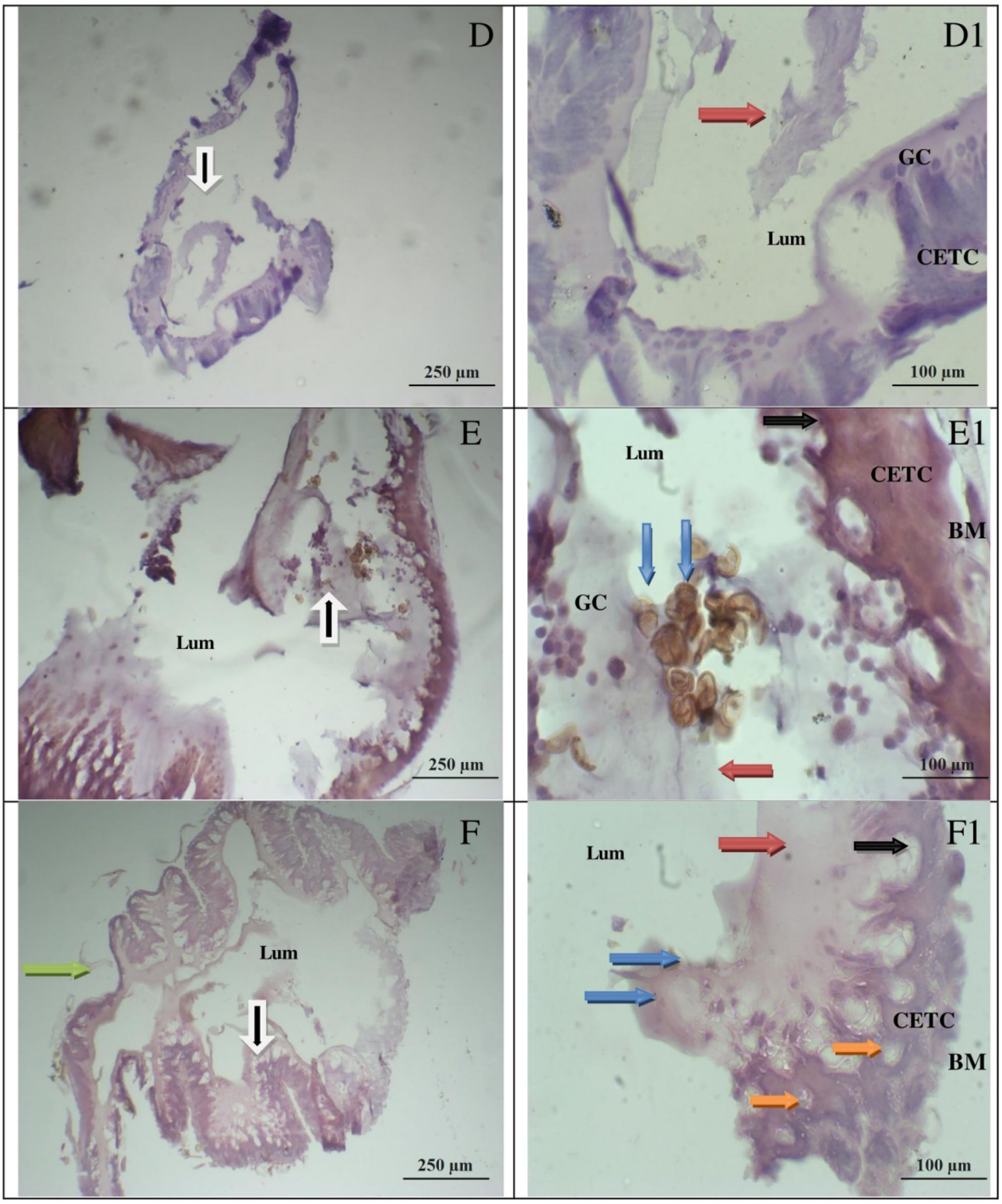

Plate 2. Experiment 5 (D, D1), Experiment 6 (E, E1), Experiment 7 (F, F1)
Green arrow: muscle layer, Red arrow: peritrophic membranes, Blue arrow: pollen wrapped in peritrophic membranes, Black and white arrow: the highlighted portion in (A1, B1 and C1), All (D, E and F) are LS of midgut. Orange arrow: cytoplasmic vacuolization, BM: basement layer, CETC: columnar epithelial cells, Lum: lumen, GC: gastric cells, Dark grey arrow: regeneration crypts

is semi-permeable and is known as the peritrophic membrane which has several functions (Cruz-Landim, 1985). These layers have the function of barrier against physical, chemical, and many food components (Terra, 1996; Terra et al., 1988). Consequently, a little bit of deformation in these layers may impart unpleasant effects on the midgut wall in insects. Deformation in the peritrophic membrane and epithelial cells of the midgut are indicators of exposure to environmental stress (Pawert et al., 1996; Zhong et al., 2012). In the current experiments, a plentiful number of peritrophic membranes was seen. Administration of organic acids, and probiotics for fourteen days, did not show any deleterious change in midgut epithelium and rabdorium, cell nuclei, and cell proliferation centers, the regeneration crypts remained unaffected. The complete

Table 1. Midgut lumen diameters of caged worker bees on provision of probiotic and organic acids.

Control	Exp 3-AA (I)	Exp 4- Lr	Exp 5-LaLr	Exp 6-AA(II)Lr	Exp 7-LrAA (I)	F- value	P-value
$240.00^{b}\pm 30.55$	$373.33^{ab}\pm 98.38$	$296.67^{b}\pm 23.33$	$243.33^{b}\pm 71.72$	$426.67^{ab}\pm 40.55$	$576.67^{a}\pm 93.87$	3.75	0.02

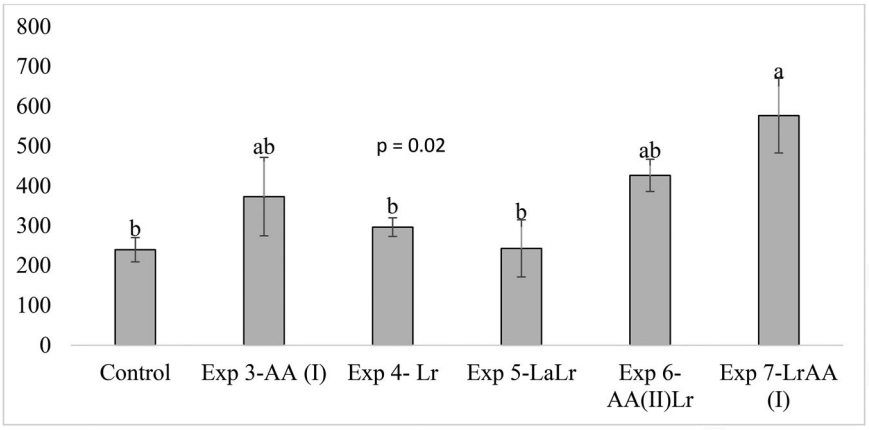

Figure 1. Midgut lumen diametersof worker bees fed with *L. rhamnosus* and organic acids.

vacuolation of cytoplasm is a sign of deformed epithelial linings (Bielenin & Ibek, 1980). Although little changes regarding cytoplasm were seen in terms of vacuolation yet the complete vacuolation of cytoplasm was not noted. The efficiency of the digestive tract gets improved in the presence of peritrophic membranes by some mechanisms (Bolognesi et al., 2008). The numerous quantities of these membranes are correlated with feeding duration. Peritrophic membranes sustain the feed in the intestine for a longer duration, so, the feed stays for a longer period in the intestine, and the more improved digestion of bees will be (Crailsheim, 1988). While discussing our treatment groups, it was found that experiments 3, 4, 5, 6, and 7 (Pollen+ 50% (w/v) sucrosein1.96% acetic acid, Pollen+ *L. rhamnosus* in 50% (w/v) sucrose in distilled water, Pollen+ *L. rhamnosus* in 50% (w/v) sucrose in 2.99% lactic acid, Pollen+ *L. rhamnosus* in 50% (w/v) sucrose in 2.91% acetic acid, Pollen+ *L. rhamnosus* in 50% (w/v) sucrose in1.96% acetic acid) had considerable quantity of peritrophic membranes.

Mean midgut lumen diameters were 373.33 ± 98.38, 296.67 ± 23.33, 243.33 ± 71.72, 426.67 ± 40.55, 576.67 ± 93.87 as compared to control's 240.00 ± 30.55. The mean midgut lumen diameter was observed largest when bees were fed on (Pollen+ *L. rhamnosus* in 50% (w/v) sucrose in 1.96% acetic acid) and it was observed smallest in the group when bees were fed with (Pollen+ *L. rhamnosus* in 50% (w/v) sucrose in 2.99% lactic acid).

Conclusions

It was observed that organic acids and probiotics administration in honey bee's feed resulted in improved health of bees and these supplements did not cause any deleterious changes in midgut wall epithelium. Moreover, it is also considered that the use of organic acids and probiotics should be patented as apipromoters.

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Disclosure statement

Q1

No potential conflict of interest was reported by the author(s)

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