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# Effect of Organic Acids and Probiotics on Growth of *Apis mellifera* Workers

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## ABSTRACT

In the present investigation, effects of organic acids and probiotics on growth of *Apis mellifera* workers were studied under different experimental conditions. Significant weight gain was observed in workers of the experimental group 8 (pollen + *Bacillus clausii* in 50 % (w/v) sucrose in distilled water), group 9 (pollen + *B. clausii* in 50 % (w/v) sucrose in 2.99 % lactic acid), group 12 (pollen + *Lactobacillus brevis* in 50 % (w/v) sucrose in distilled water) and group 14 (pollen + *L. brevis* in 50 % (w/v) sucrose in 2.91 % acetic acid). The weight gain values (mg) for the experimental groups 8, 9, 12 and 14 appeared as 138.87 ± 6.50, 131.50 ± 4.35, 124.08 ± 5.28 and 127.82 ± 2.32, respectively in comparison to control's 119.90 ± 9.50. Significant increase in body lengths of workers in the experimental groups 8, 9 and 11 (pollen + *B. clausii* in 50 % (w/v) sucrose in 1.96 % acetic acid) showing mean length values (mm) as 15.33 ± 0.67, 15.75 ± 0.25 and 15.33 ± 0.33, respectively in comparison to control's 14.67 ± 0.33. Similarly, somewhat unexpected, and significant increase in forewing length was also noticed while observing the workers of treatment groups 8 and 12 (pollen + *L. brevis* in 50 % (w/v) sucrose in distilled water) showing forewing length values (mm) as 6.75 ± 0.09 and 6.55 ± 0.04, respectively in comparison to control's 6.53 ± 0.09. Workers belonging to the experimental groups 1 to 7 depicted insignificant results. Conclusively, api-promotor properties of organic acids and probiotics recommend their use in modern honeybee feeds.

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**AH performed all experiments. JIQ supervised the work. NM worked on collecting and arranging data. AH drafted manuscript. SJ helped in statistical analysis and compilation of data.**

**Key words**
**Bee farming, Beneficial bugs, Feed formulation, Growth promotors, Modern apiculture**



## INTRODUCTION

*Apis mellifera* is an important pollinator having prominent impact on the ecological balance and economy of crops. Therefore, identification of different factors affecting honeybees' health is important for attaining proper ecological balance and high yield of crops. Different factors have negative impacts on colony bees as well as health including pathogens (Cox-foster *et al*., 2007), pesticide exposure, parasites (Sanchez-Bayo and Goja, 2014), poor nutrition (Brodschneider and Crailsheim, 2010) and it may include the interactions of the factors described above (Potts *et al*., 2010). Environment may pose significant effect on body size and health condition of honeybees exactly like that nutrition and temperature pose drastic effects on different species of invertebrates (Partridge *et al*., 1994; Metcalfe and Monaghan, 2001; Angilletta *et al*., 2004; Cassidy *et al*., 2014; Scofield and Mattila, 2015). Free living organisms developing in stochastic and variable environments have probability to suffer conditions of non-ideal development (Awmack and Leather, 2002). For instance, in comparison, honeybee workers grown *in-vitro* show morphological distinctions with the workers grown in hives (Brodschneider *et al*., 2009; Kaftanoglu *et al*., 2010).

The health of honeybee colony can be improved with the use of probiotics and prebiotics. On the other hand, acidifying agents (organic acids) lower down the pH and act like antimicrobials hence prohibiting the growth of pathogenic bacteria. It has been observed experimentally that bees fed with sugar syrup having pH between 3−6.5 showed maximum suppression in pathogenic bacterial growth. Various investigations have proved that probiotics not only restore digestive dysfunction but also exert important effects in inhibition of pathogenic bacterial colonization and improvement of host immunity (Patruica *et al*., 2013). Furthermore, probiotics have contribution in establishing stable and appropriate environment of bacteria in honeybees' gut (Kaznowski *et al*., 2005).

Four well-known honeybee species (*A. mellifera*, *A. dorsata*, *A. cerana* and *A. florea*) are found in Pakistan. *A. mellifera* was brought to the country in 1977 for commercial beekeeping (Hussain *et al*., 2015). A typical honeybee colony consists of a queen, male drones in hundreds and female worker bees in thousands (Khan *et al*., 2016). Keeping in view the importance of honeybees

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in the natural environment and for sustainable modern apiculture, the present study was designed to explore the hidden potential of organic acids (lactic acid and acetic acid) and probiotics (*Lactobacillus rhamnosus*, *Lactobacillus brevis* and *Bacillus clausii* spores) on degree of development of different body parts of *A. mellifera* workers.

## MATERIALS AND METHODS

*Ethical statement*

The research work needed no specific permit, and we conducted all our experiments in The Institute of Zoology, University of the Punjab, Lahore, Pakistan. Bees for the experiments were taken from Honeybee Garden (31.49758º N; 074.29679 E). The apiary is a property of The Institute of Zoology, University of the Punjab, Lahore, Pakistan. Honeybee Garden is not protected in any way. In addition, *A. mellifera*, the honeybee species which was used in our study is not protected species or endangered in Pakistan.

*Honeybee workers and experimental setup*

Newly emerged *A. mellifera* workers of 0−24 h age were secured by incubating beehives having large number of sealed broods at 34°C after Williams *et al*. (2013). Honeybee combs selected for getting the brood were from many unrelated colonies. Frames selected for incubation had pupae with dark eyes and brownish cuticle and usually emerged from the cells within 1 to 2 days. Frames were relatively new, and their color was not dark enough and were not grubby with honeybees' fecal material and had little amount of stored food also. As there was not enough space to compensate full frame in the incubator therefore portions of frames with affluent amount of capped brood were secured and incubated. A bowl was filled with tap water and covered with mesh before placing in the incubator to maintain humidity according to Williams *et al*. (2013) with slight modification. Bowl was covered with a mesh to save newly emerging bees from being drowned in the bowl. After 24 h, newly emerged bees were collected from the hive portions by gentle brushing to hoarding cages for different treatments.

The experiments were conducted in plastic cages measuring 11 cm × 6 cm × 6 cm. The jars were fully transparent and hence it was quite easy to observe behavior of the bees. These plastic jars were extensively perforated by heated solder iron with pore size was of about (2 mm). The dead honeybees were removed on daily basis from the cages. All the cages were fitted with feeders and pollen providers. Pollen providers were made from Eppendorf tubes of 2 mL capacity by removing their bottoms. Sugar syrup was provided to the caged *A. mellifera* workers in 5 mL syringe having pore diameter of 3 mm.

*Probiotics' culturing and dose preparation*

Three different kinds of probiotic bacterial species were employed in the present study. Description of the probiotics used in this study is given in Table I. Sterile MRS broth (Biolife, Italiana) was inoculated with *L. rhamnosus* culture under aseptic conditions and a thick layer of autoclaved liquid paraffin was poured on the surface of the bottle having inoculated broth to maintain anaerobic conditions. The culture bottle was then incubated at 37 oC. Growth of cells like upraising cigarette smoke was seen between 24−48 h incubation. The growth of cells was measured with the help of V-M5 VIS-spectrophotometer.

**Table I. Sources of probiotics administered to the experimental bees.**

| Probiotic | Source | C.F.U. $m^{-1}$ of sugar syrup |
|---|---|---|
| *L. rhamnosus* (NR_113332) | Microbial Biotechnology Laboratory, Institute of Zoology | $1 \times 10^8$ |
| *B. clausii* spores (BC-2Bio) | Purchased from local market (TABROS pharma) | $1 \times 10^8$ |
| *L. brevis* (MF179529) | Immunology Laboratory, Institute of Zoology | $1 \times 10^8$ |

*Estimation of C.F.U. $mL^{-1}$ of MRS broth*

Broth culture of *L. rhamnosus* was serially diluted with sterile distilled water. For this purpose, 15 capped test tubes containing 9 mL of the distilled water were autoclaved at 15 psi and 121 ºC for 20 min. After cooling, 1 mL of broth containing massive growth was transferred to test tube labeled as one, and then it was serially diluted to test tube 15. Then 100 µL from each dilution was spread on MRS agar plate and 1 mL was also taken quickly from each dilution tube to check its optical density. It was found that $1 \times 10^8$ (C.F.U. $mL^{-1}$) of *L. rhamnosus* showed optical density of 0.5 at (600 nm) using V-M5 VIS-spectrophotometer. Thereafter, MRS broth of the given O.D. was diluted up to 0.5 readings by pouring autoclaved fresh MRS broth in culture bottle gradually. After attaining 0.5 O.D., the culture was centrifuged to have C.F.U. of $1\times 10^8$ $mL^{-1}$. All the above-described procedure was accomplished in laminar air flow cabinet.

*Probiotic dose preparation as master stock*

Fresh MRS broth was inoculated, and autoclaved liquid paraffin was poured on top of the culture bottle to maintain anaerobic condition. After 24−48 h post incubation period, the layer of liquid paraffin was removed from the surface of broth carefully using micropipette of 1,000−5,000 µL capacity. Then 1 mL culture was taken in cuvette for optical density measurement using V-M5

VIS-spectrophotometer. The fresh MRS broth was used as blank. After taking O.D. readings, the culture was diluted down to 0.5 O.D. The culture bottle was shaken thoroughly to suspend the cells evenly and then 2 mL of the broth (having O.D. of 0.5) from culture bottle was taken in Eppendorf tubes (2 mL capacity) and centrifuged at 10,000−15,000 rpm and 25 °C to make pellet of cells. After centrifugation, supernatant was discarded, and pellet of cells was saved in physiological saline. This master stock of probiotic dose was preserved in a refrigerator (at about 7 °C) till future use. The above-mentioned procedure was adopted for the bacterial species *L. rhamnosus* and *L. brevis*, while in case of *B. clausii* (an industrially available probiotic, TABROS pharma), each packing contained 2 billion C.F.U. 5 $mL^{-1}$. Each packing of 2 billion C.F.U. was equally divided in 5 parts in Eppendorf tubes (each containing $4 \times 10^8$ C.F.U. $mL^{-1}$) and after centrifugation at 10,000−15,000 rpm supernatant was discarded and pellet of cells was saved in physiological saline and stored in a refrigerator as described before.

*Serving corbicular pollen to the experimental caged workers*

To provide protein source to the caged honeybees, 9 g of carbicular pollens were mixed with 1 mL of water in a beaker and kneaded well with gloved finger and metallic spatula to create thick paste whose consistency was similar to soft dough according to Alaux *et al*. (2010).

*Provision of sugar syrups and probiotics to the caged honeybees*

Sugar syrup was prepared by mixing sucrose in distilled water following Martín-Hernández *et al*. (2011). The control group was provided with only sugar syrup, while the experimental groups were provided with probiotic-added sugar syrup. Stock solutions of organic acids were prepared by mixing 0.75 mL of lactic acid per 250 mL of distilled water and 7.5 mL acetic acid per 250 mL of distilled water following Patruica *et al*. (2013). To explore the effects of organic acids alone or in combination form with probiotics, sugar syrup was prepared by mixing stock solutions of organic acids with sucrose in 1:2 ratios instead of using distilled water. The pH detail of sugar syrups provided to each group is described in Table II.

*Daily feeding of honeybees*

In the present experiments, $1 \times 10^8$ C.F.U. $mL^{-1}$ and sugar syrup were provided. Four milliliters of sugar syrup were provided to a cage daily. For the probiotics supplemented groups, $1 \times 10^8$ C.F.U. $mL^{-1}$ of a respective probiotic bacterial species were blended per mL of the sugar syrup and fed to the experimental group daily after Szymas *et al*. (2012) with little bit modifications of number of C.F.U. and vehicle. As bees were provided with 4 mL sugar syrup on daily basis, so two Eppendorf tubes having $4 \times 10^8$ C.F.U. $mL^{-1}$ were withdrawn from master stock of probiotics and centrifuged at 10,000-15000 rpm at 25 °C for 10 min. The supernatant was discarded carefully, and pellet of cells was socked in little quantity of sugar syrup already prepared on that day. By closing the lid of Eppendorf tube again, the contents of the Eppendorf were blended with the help of vortex mixer till the formation of suspension of cells and sugar syrup. Syringe feeder was set at 4mL quantity sign and the mixtures from Eppendorf tubes were shifted to syringe feeder by the aid of sterile syringe and by using the same syringes (used to pick suspensions of pellet cells and sugar syrups) the remaining volume of feeders were filled. This procedure was repeated for each probiotic. Detail of the experimental design is shown in Table II.

**Table II. Description of experimental and control groups.**

| Experimental group | Description (Pollen+) |
|---|---|
| ***L. rhamnosus* with organic acids** | |
| Control I: 50 % sucrose in DW (pH: 8) | |
| 1 | 50 % sucrose in 2.99 % LA (pH: 3.14) |
| 2 | 50 % sucrose in 2.91 % (AA pH: 2.95) |
| 3 | 50 % sucrose in1.96 % AA (pH: 3.12) |
| 4 | *L. rhamnosus* in 50 % sucrose in DW (pH: 8) |
| 5 | *L. rhamnosus* in 50 % sucrose in 2.99 % LA (pH: 3.14) |
| 6 | *L. rhamnosus* in 50 % sucrose in 2.91 % AA (pH: 2.95) |
| 7 | *L. rhamnosus* in 50 % sucrose in 1.96 % AA (pH: 3.12) |
| ***B. clausii* and *L. brevis* with organic acids** | |
| Control II: 50 % sucrose in DW (pH: 8) | |
| 8 | *B. clausii* in 50 % sucrose in DW (pH: 8) |
| 9 | *B. clausii* in 50 % sucrose in 2.99 % LA (pH: 3.14) |
| 10 | *B. clausii* in 50 % sucrose in 2.91 % AA (pH: 2.95) |
| 11 | *B. clausii* in 50 % sucrose in 1.96 % AA (pH: 3.12) |
| 12 | *L. brevis* in 50 % sucrose in DW (pH: 8) |
| 13 | *L. brevis* in 50 % sucrose in 2.99 % LA (pH: 3.14) |
| 14 | *L. brevis* in 50 % sucrose in 2.91 % AA (pH: 2.95) |
| 15 | *L. brevis* in 50 % sucrose in 1.96 % AA (pH: 3.12) |

AA, acetic acid; DW, distilled water; LA, lactic acid.

*Collection and dissection of bees for growth analysis*

Bees were placed at −20 °C in a freezer for 2 h following Naug and Gibbs (2009) as placing bees under these conditions is usually sufficient to kill them.

*Morphometric analyses of bee worker's body parts*

Nine characters pertaining body weight, body

length, forewing length, forewing width, hindwing length, hindwing width, femur length, tibia length and width of hind metatarsus were measured. Body length was measured using millimeter scale, while other measurements were carried out with stage micrometer of Erma optics. The specimens were placed on stage micrometer and observed under stereomicroscope (ER-59-1828, Carolina Biological Supply Company). Another clean slide was used to cover the specimen on stage micrometer to remove the curve in case of wings. The obtained values were converted into millimeter scale.

*Statistical analysis*

The data were analyzed according to completely randomized design (CRD) under factorial arrangement using general linear model (GLM) procedures. Means were separated out using Duncan's multiple range (DMR) test with the help of SAS 9.1 for windows. Differences between means were considered significant at $P < 0.05$.

## RESULTS

Control bees (I) which were fed with pollen and sugar syrup for two weeks depicted the mean values of 118.66 mg ± 9.57, 13.00 mm ± 0.45, 9.29 mm ± 0.07, 3.13 mm ± 0.01, 6.60 mm ± 0.06, 1.80 mm ± 0.001, 2.35 mm ± 0.02, 2.94 mm ± 0.04, 1.03 mm ± 0.03 for body weight, body length, forewing length, forewing width, hindwing length, hindwing width, femur length, tibia length and metatarsus width, respectively (Table III).

Following two weeks of acidifying agents' doped feeding (experiments 1 and 3), the mean ± SE values for nine characters were body weight (124.45 mg ± 8.59, 126.50 mg ± 11.35), body length (14.00 mm ± 0.41, 14.25 mm ± 0.48), forewing length (9.18 mm ± 0.21, 9.15 mm ± 0.10), forewing width (3.14 mm ± 0.06, 3.13 mm ± 0.03), hindwing length (6.65 mm ± 0.09, 6.65 mm ± 0.15), hindwing width (1.86 mm ± 0.05, 1.88 mm ± 0.01), femur length (2.47 mm ± 0.06, 2.41 mm ± 0.01), tibia length (2.99 mm ± 0.05, 2.96 mm ± 0.04 ) and metatarsus width (1.00 mm ± 0.03, 1.05 mm ± 0.03). Comparison of mean values of all the acid-treated bees with control group revealed that honeybees of the experimental group 1 showed reasonable increase in all characters except metatarsus width and forewing length, while in case of the experiment group 2, honeybees showed reasonable increase in all the characters except forewing length, forewing width and metatarsus width as shown in Table III.

Morphometric measurements showing growth of the bees fed on diets supplemented with *L. rhamnosus* and organic acids under the experimental groups 4 to 7 are shown in Table III. It was observed that all the characters in all treatment groups showed reasonable increases except forewing length (experimental groups 1, 3, 5 and 7), forewing width (experimental groups 4, 5 6), hindwing length (experimental groups 4, 6 and 7), tibia length (experimental groups 4, 5 and 7), metatarsus width (experimental groups 1, 4 and 5) as evident from Table III.

Another batch of control bees **(II)** which were fed with pollen and sugar syrup for two weeks depicted the mean values of 119.90 mg ± 9.50, 14.67 mm ± 0.33, 9.43 mm ± 0.03, 3.08 mm ± 0.02, 6.53 mm ± 0.09, 1.85 mm ± 0.03, 2.38 mm ± 0.02, 2.97 mm ± 0.03 and 1.02 mm ± 0.02 for body weight, body length, forewing length, forewing width, hindwing length, hindwing width, femur length, tibia length and metatarsus width, respectively (Table IV).



**Table III. Morphometric measurements obtained after different treatments of organic acids and *L. rhamnosus*.**

| Parameter | Control group | Experimental groups | | | | | | P-value | D.F |
|---|---|---|---|---|---|---|---|---|---|
| | | 1 | 3 | 4 | 5 | 6 | 7 | | |
| BW (mg) | 118.66±9.57 | 124.45±8.59 | 126.50±11.35 | 128.20±7.37 | 135.20±5.60 | 140.90±7.51 | 124.60±3.12 | 0.4969 | 6 |
| BL (mm) | 13.00 ± 0.45 | 14.00 ± 0.41 | 14.25 ± 0.48 | 13.50 ± 0.50 | 14.60 ± 0.51 | 14.50 ± 0.29 | 14.40 ± 0.24 | 0.0977 | |
| FWL (mm) | 9.29 ± 0.07 | 9.18 ± 0.21 | 9.15 ± 0.10 | 9.40 ± 0.001 | 9.20 ± 0.11 | 9.38 ± 0.13 | 9.04 ± 0.15 | 0.4128 | |
| FWW (mm) | 3.13 ± 0.01 | 3.14 ± 0.06 | 3.13 ± 0.03 | 3.10 ± 0.04 | 3.08 ± 0.05 | 3.05 ± 0.03 | 3.14 ± 0.02 | 0.5591 | |
| HWL (mm) | 6.60 ± 0.06 | 6.65 ± 0.09 | 6.65 ± 0.15 | 6.55 ± 0.03 | 6.60 ± 0.08 | 6.58 ± 0.05 | 6.54 ± 0.07 | 0.9398 | |
| HWW (mm) | 1.80 ± 0.001 | 1.86 ± 0.05 | 1.88 ± 0.01 | 1.83 ± 0.02 | 1.79 ± 0.04 | 1.83 ± 0.02 | 1.83 ± 0.03 | 0.4632 | |
| FL (mm) | 2.35 ± 0.02 | 2.47 ± 0.06 | 2.41 ± 0.01 | 2.30 ± 0.04 | 2.35 ± 0.05 | 2.36 ± 0.06 | 2.45 ± 0.02 | 0.0700 | |
| TL (mm) | 2.94 ± 0.04 | 2.99 ± 0.05 | 2.96 ± 0.04 | 3.03 ± 0.03 | 2.96 ± 0.09 | 2.85 ± 0.09 | 3.01 ± 0.04 | 0.5349 | |
| MTW (mm) | 1.03 ± 0.03 | 1.00 ± 0.03 | 1.05 ± 0.03 | 0.98 ± 0.03 | 1.00 ± 0.03 | 1.05 ± 0.02 | 1.03 ± 0.01 | 0.4537 | |

BW, Body weight; BL, Body length; FWL, Forewing length; FWW, Forewing width; HWL, Hindwing length; HWW, Hindwing width; FL, Femur length: TL, Tibia length; MTW, Metatarsus width; D.F, Degrees of freedom. Values are means ± S.E. of three replicates.

**Table IV. Morphometric results obtained after different treatments of *L. brevis* and *B. clausii* with organic acids.**

| Parameter | Control group | Experimental groups | | | | | | | P value | D.F |
|---|---|---|---|---|---|---|---|---|---|---|
| | | 8 | 9 | 11 | 12 | 13 | 14 | **15** | | |
| BW (mg) | $119.90^{bcd}$ ±9.50 | $138.87^{a}$ ±6.50 | $131.50^{ab}$ ± 4.35 | $105.67^{d}$ ± 2.60 | $124.08^{abc}$ ± 5.28 | $119.28^{bcd}$ ± 3.10 | $127.82^{abc}$ ± 2.32 | $114.50^{cd}$ ± 4.92 | 0.0064 | 7 |
| BL (mm) | $14.67^{bc}$ ±0.33 | $15.33^{ab}$ ±0.67 | $15.75^{a}$ ± 0.25 | $15.33^{ab}$ ± 0.33 | $14.60^{bc}$ ± 0.24 | $14.20^{c}$ ± 0.20 | $14.40^{bc}$ ± 0.24 | $14.50^{bc}$ ± 0.29 | 0.0140 | |
| FWL (mm) | 9.43 ± 0.03 | 9.63 ± 0.07 | 9.20 ± 0.04 | 9.28 ± 0.15 | 9.26 ± 0.09 | 9.37 ± 0.09 | 9.27 ± 0.08 | 9.35 ± 0.09 | 0.1146 | |
| FWW (mm) | 3.08 ± 0.02 | 3.12 ± 0.02 | 3.06 ± 0.02 | 3.08 ± 0.04 | 3.12 ± 0.02 | 3.06 ± 0.02 | 3.07 ± 0.03 | 3.05 ± 0.03 | 0.4861 | |
| HWL (mm) | $6.53^{abc}$ ±0.09 | $6.75^{a}$ ±0.09 | $6.30^{bc}$ ±0.15 | $6.27^{c}$ ± 0.09 | $6.55^{abc}$ ±0.04 | $6.47^{abc}$ ±0.09 | $6.43^{bc}$ ±0.09 | $6.61^{ab}$ ± 0.09 | 0.0527 | |
| HWW (mm) | 1.85 ± 0.03 | 1.85 ± 0.05 | 1.85 ± 0.04 | 1.82 ± 0.03 | 1.87 ± 0.02 | 1.85 ± 0.02 | 1.85 ± 0.03 | 1..81 ± 0.03 | 0.8889 | |
| FL (mm) | 2.38 ± 0.02 | 2.47 ± 0.03 | 2.44 ± 0.06 | 2.42 ± 0.03 | 2.45 ± 0.02 | 2.45 ± 0.03 | 2.45 ± 0.04 | 2.49 ± 0.03 | 0.7391 | |
| TL (mm) | 2.97 ± 0.03 | 3.10 ± 0.03 | 3.06 ± 0.04 | 2.97 ± 0.04 | 3.04 ± 0.03 | 3.00 ± 0.05 | 3.01 ± 0.02 | 3.04 ± 0.03 | 0.3028 | |
| MTW (mm) | 1.02 ± 0.02 | 1.07 ± 0.03 | 1.03 ± 0.01 | 1.02 ± 0.03 | 1.05 ± 0.02 | 1.01 ± 0.02 | 1.02 ± 0.04 | 1.04 ± 0.02 | 0.8043 | |

For abbreviations, see Table III.
Those not sharing a common alphabet within a respective column are significantly different from each other.

Honeybees fed on diets supplemented with *B. clausii* without and with organic acids, i.e., experimental groups 8, 9, 10 and 11 depicted interesting results. It was observed that body weight and body length increased significantly in case of the experimental groups 8 and 9, while all other characters in all treatment groups showed reasonable increases except femur width and length (experimental groups 9 and 11), forewing width (experimental group 9) and hindwing length (experimental groups 9 and 11) as shown in Table IV. Bees of the experimental group 8 showed best growth results in terms of morphometric characters (Table IV).

Experimental groups fed on diets doped with *L. brevis* with and without organic acids, i.e. experimental groups 12−15 also depicted variations in different growth characters. For the experimental groups 12 and 14, significant increase in body weight of bee workers was recorded. All other characters in the experimental groups (12−15) showed reasonable increase except body weight (experimental groups 13 and 15), forewing width (experimental groups 13, 14 and 15) and metatarsus width and hindwing width in the experimental groups 13 and 15 (Table IV). Body and forewing lengths were increased in all these experimental groups.

## DISCUSSION

Due to the development of bacterial resistance to different antibiotics and ultimate ban on them for beekeeping sector in Europe, the use of probiotics has become need of the hour. Numerous studies insist the use for probiotics to improve workers as well as entire colony health because it is environment friendly methodology for rehabilitation of the host. The purpose of study was to evaluate the contribution of probiotics and organic acids in the bee development under controlled experimental conditions. The degree of development of honeybees was judged by taking morphometric measurements. Morphometric analyses have been documented to exhibit correlation to honey yield (Kolmes and Sam, 1991). The morphometric nine characters of honeybee have critical role in colony productivity and honey yield. Morphometrics help in predicting the productivity of colony in such a way that honeybees having big sized wings and legs have ability to gather more quantity of nectar and pollen for colony population and brood rearing (Mostajeran *et al*., 2006). Similarly, lengths of fore and hind wings, width of fore and hind wings, tongs' length, tibia, femur, and metatarsus have been related with honey production. Our results regarding development of all bee appendages are supported by the above-mentioned statements. We observed that our treatment groups resulted in reasonable increases in honeybee's body characters like forewing, hind wing, femur length, tibia length and metatarsus width. It was found that lactic acid (2.99 %) and acetic acid (experimental groups 1 and 3) resulted in reasonable increases in all the studied body characters. Administration of *L. rhamnosus* (experimental group 7), *B. clausii* (experimental group 11) and *L. brevis* (experimental group 15) resulted in decreased growth. When probiotics were administered in the absence of acidifying agents it was found that *B. clausii* (experimental group 8) showed better growth results than *L. brevis* (experimental group 12) and *L. rhamnosus* (experimental group 4). During spring to summer, the area for foraging is increased that may be much challenging for *A. mellifera* workers to search for food.

Therefore, it may be advantageous for honeybee colonies to generate large sized workers as reported by Couvillon *et al*. (2014). Provision of *L. rhamnosus* with organic acids (experimental group 1 to 7), *L. brevis* and *L. brevis* with acetic acid (experimental groups 12, 14 and 15), *B. clausii* and *B. clausii* with lactic acid (experimental groups 8 and 9) showed reasonable increase in body weights. It is known that large sized workers have ability to depart away the nest for foraging (Kapustjanskij *et al*., 2007). In our experiments, significant increases in body lengths of worker honeybees were found in groups provided with *B. clausii* in 1.96 % acetic acid (experimental group 11), *B. clausii* in 2.99 % lactic acid (experimental group 9) and *B. clausii* (experimental group 8). Further behavioral investigations addressing worker duties i.e., they either tend to stay at nest or prefer it to be foragers may dig out further relevant information following varying feeding experimentations including administration of probiotics and/or organic acids.

## CONCLUSIONS AND RECOMMENDATIONS

Our study endorsed the statement that organic acids and probiotics impart positive effects on honeybees regarding their growth and development. The environment-friendly methodology can be patented and promoted for improving our beekeeping sector. We recommend the use of probiotics and organic acids for better improvement of *A. mellifera* workers.

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*Statement of conflict of interest*

The authors have declared no conflict of interest.

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