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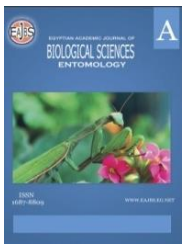

Antibacterial Effect of Emerging Queen Honeybee Induced by The Bacterium *Paenibacillus* Larvae Subsp. Against Multidrug-Resistant Bacteria

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ABSTRACT

This study investigated the antimicrobial activity of body and ovary homogenates of emerging queens of the honeybee *Apis mellifera* L. Queens'. Fourth-instar larvae were challenged with a sublethal dose of *Paenibacillus larvae* to investigate the effect against multidrug-resistant bacteria (*Escherichia coli* 1 and 2 and *Klebsiella pneumoniae*) and the causative agent of American Foulbrood (*Paenibacillus l. l*). The agar disk diffusion test was used. Against the selected pathogenic bacteria, both the body and the ovary homogenates showed antibacterial activity. A highly significant change in the antibacterial activities of total body homogenate was detected for treated queens against *E. coli 2* as well as *K. pneumoniae* (0.65 ± 0.02 and 0.65 ± 0.01, respectively) compared with healthy queens (0.45 ± 0.02 and 0.45 ± 0.02, respectively). On the other hand, the ovary extracts of challenged queens showed highly significant increases in antibacterial activity against *P. l. larvae*, *E. coli* 1, and *K. pneumonia* (0.92 ± 0.05, 0.85 ± 0.4, and 0.67 ± 0.0004, respectively) compared with the healthy queens (0.55 ± 0.02, 0.65 ± 0.23, and 0.49 ± 0.001, respectively). These findings could provide us with essential points on the development and production of new antibiotic agents against bacterial diseases.

INTRODUCTION

To fight infections, insects rely on their inducible systemic humoral immune system, which produces a battery of antimicrobial peptides (AMPs) in response to infection by bacteria, fungi, or parasites (Yamauchi, 2001; Klaudiny *et al*., 2005). Upregulation of various AMPs is known to occur in response to oral bacterial infections (Evans, 2004; Kačániová *et al.*, 2018). Various AMPs are synthesized and secreted in the fat body (Angus *et al*., 2001), and released into the hemolymph, forming a general nonspecific line of defense. Honeybee AMPs consist of at least four peptides, namely, apidaecin (Casteels *et al*., 1989), abaecin (Casteels *et al*., 1990), hymenoptaecin (Casteels *et al*., 1993), and defensin (Casteels-Jonsson *et al*., 1994), each of which has a rather broad spectrum of activity (Tzou *et al*., 2002). All peptides were found to show inhibitory activity against bacteria *in vitro* (Casteels-Josson *et al*., 1994). Another identified AMP is

vitellogenin, which is synthesized by the fat body and released in the female hemolymph, having a bactericidal effect (Zhang *et al.,* 2011). AMPs are low-molecular-weight proteins with broad-spectrum antimicrobial activity against different environmental pathogens (Guaní-Guerra *et al.,* 2010; Da Silva and Machado, 2012). These peptides are usually positively charged, enabling them to be soluble in aqueous conditions and permeate lipid-rich membranes for rapid infection (Aerts *et al*., 2008).

Mayers *et al.* (2009) developed the agar disk diffusion method, which is the method for routine antimicrobial testing in many clinical laboratories (Balouiri *et al*., 2016). After inoculating agar plates with the tested microorganism's inocula, filter paper disks containing the test compound at a certain concentration are placed on the agar surface. The plates are incubated under suitable conditions, where the antimicrobial agent diffuses into the agar and inhibits the growth of the tested microorganism. The zone with growth inhibition is then measured. This provides qualitative results by classifying bacteria as resistant, intermediate, or susceptible (Jorgensen and Ferraro, 2009). This approach is simple to use, inexpensive and provides researchers with the ability to test a wide range of microorganisms and antimicrobial agents.

Multidrug-resistant pathogens have been found all over the world in large numbers. Currently available antibiotics are unable to treat these multidrug-resistant infections. As such, there is a global push to find antibiotic alternatives (Farmanullah *et al*., 2020; Abou Nader *et al*., 2021). To minimize the threat posed by drug-resistant bacteria to public health, new antimicrobial drugs are currently being developed. Our research attempts to contribute new information regarding the effect of AMPs of queen honeybee larvae on multidrug-resistant bacteria.

MATERIALS AND METHODS

Source and Rearing of Honeybees:

This study used two colonies of Craniolian hybrid honeybees (*Apis mellifera carnica*) in a private apiary. Tested queens were obtained using the grafting technique (Doolittle, 1889), which is used for rearing queens to produce a large number of them on a commercial scale. Routine methods of keeping and developing the colonies were carried out during the experimental period. The total developmental period of the bred honeybee queens was estimated at nearly 16 days. The honeybee brood frame containing 1-day-old larvae was selected. The selected larvae were transferred to cups fixed on horizontal wooden bars in a special grafting frame. Three days later, the cup cells containing the fourth-instar larvae were treated with a sublethal dose of *P. l. larvae* bacteria or treated with water (positive control), via the queens' food.

Source of the Bacterial Pathogen:

The bacterium used in this study, *Paenibacillus larvae larvae*, was isolated from ropy remains of honeybee larvae collected from the Agriculture Research Center, Plant Protection Institute, Department of Apiculture Research. Ropy larval remains of dead honeybee larvae (collected from infected colonies) were suspended in 10 ml of sterile distilled water and kept at room temperature for 10 min, after which the suspension was heat-shocked at 80ºC for 15 min (effective time to kill non-spore-forming bacteria). The J-agar medium was prepared by mixing 900 ml of distilled water with 5.0 g of tryptone, 15.0 g of yeast extract, 3.0 g of K_2HPO_4, 2.0 g of glucose, and 20.0 g of agar; then, the pH was adjusted to 7.3–7.5 and distilled water was added up to a volume of 1 L (Shimanuki and Knox, 1988). The mixture was autoclaved at 121ºC and 1 atm for 15 min. The bacterial slants were refreshed (renewed) using J-agar slants, incubated to become active (vegetative cells) *P. l. larvae* bacteria, and then stored at 4ºC. Different

multidrug-resistant bacteria (*Escherichia coli* 1 and 2 and *Klebsiella pneumoniae*) were isolated from previous studies by Mekkawy (2017) and identified using Vitek 2.

Immunization of Honeybee Queens Induced by A Bacterium, *P. l. larvae*:

In our previous investigation, a stock suspension of a sublethal dosage of *P. l. larvae* (10^7 CFU/queen) was established (Gomaa *et al.*, 2021). It was used for inoculation by adding 10 µl of bacterial suspension to the food of a group of honeybee queens at the fourth-instar larval stage (Decanini *et al.*, 2007). Two groups of controls were used: the first was fourth-instar larvae of queens the food of which had been treated with 10 µl of autoclaved distilled water (positive control), while the second group was healthy untreated honeybee queen larvae (negative control).

Total Body Homogenate:

Healthy, water-treated, and bacterially treated adult queens were collected after emergence. They were crushed in a sterile Eppendorf tube using Ultrasonic Homogenizer, 4710 Series, at 30 Hz for 2 min on ice, after which 250 µl of phosphate-buffered saline (PBS) (OXOID) was added and pipetted well. The samples were then centrifuged (Eppendorf Centrifuge, 5402) at 4000 rpm for 15 min upon cooling. The obtained supernatant was decanted into another sterile Eppendorf tube and stored at –20ºC.

Ovary Extract:

Healthy, water-, and bacterially treated adult queens were dissected and their ovaries were collected. The ovaries were crushed in a sterile Eppendorf tube using a sterilized bristle on ice, after which 100 µl of PBS was added and pipetted well. The samples were then centrifuged (Eppendorf Centrifuge, 5402) at 5000 rpm for 15 min upon cooling; the supernatant was decanted into another sterile Eppendorf tube and stored at –20ºC until used.

Assaying of Antibacterial Activity:

Tests were conducted to determine whether the total body homogenate and ovary extracts exhibited antibacterial activity. Supernatants of bacterially treated queens were assayed and compared with those of controls. The agar disk diffusion test was used by the work of Heatley (1944). The plates of media were inoculated with 1 ml bacterial suspensions (*P. l. larvae*, *E. coli* 1 and 2, and *K. pneumoniae*) on the agar surface. Then, sterilized filter paper disks (about 6 mm in diameter) containing 10 µl of each honeybee queen's testing sample were placed in the center of the Petri dishes with agar bacterial medium. The Petri dishes were incubated under the conditions shown in Table 1. Antimicrobial agents diffused from the queens' samples into the agar. The inhibition of germination and growth of the tested microorganisms was investigated and then the diameters of the inhibition zones were measured in millimeters with a ruler. The measurements were repeated five times for each sample.

Table 1. Types of bacteria are used to determine the antibacterial activity of emerging honeybee queens.

Bacteria	Gram stain	Isolation location	Importance	Medium used	Temperature and incubation period
Paenibacillus larvae larvae	+ve	Infected honeybee larvae	Fatal honeybee brood disease	J-agar	35°C–37ºC 24 h
Escherichia coli 1	+ve	Blood isolate	Multidrug resistance	Brain heart infusion agar	30°C–34ºC 24 h
Escherichia coli 2	+ve	Throat swab	Multidrug resistance	Brain heart infusion agar	30°C–34ºC 24 h
Klebsiella pneumoniae	+ve	Throat swab	Multidrug resistance	Brain heart infusion agar	30°C–34ºC 24 h

Data Analysis:

Data are expressed as mean ± standard error (SE). The significance of differences of means was determined using Student's *t*-test for paired samples.

RESULTS

Antibacterial activity of the body homogenate

The bactericidal activity of total body homogenate against the bacterium *P. l. larvae* and multidrug-resistant bacteria is shown in Figure 1. The mean dimensions of the inhibition zones against *P. l. larvae* were 0.82 ± 0.06, 1.3 ± 0.07, and 0.84 ± 0.05 cm in healthy queens, water-fed queens, and bacterially fed queens, respectively. The antibacterial activity of bacterially treated queens did not differ significantly ($P > 0.05$) from that of healthy queens but was significantly decreased ($P \leq 0.01$) compared with that of the water-fed group. The mean dimensions of inhibition zones of healthy, water-fed, and treated queens against *E. coli* 1 were (1.3 ± 0.12, 1.5 ± 0.2, and 1.25 ± 0.05 cm, respectively). In *E. coli* 2, the zones were (0.45 ± 0.02, 0.92 ± 0.2, and 0.65 ± 0.02 cm) for healthy, control, and treated queens, respectively. Inhibition zone dimensions of *K. pneumoniae* were (0.45 ± 0.02, 0.5 ± 0.01, and 0.65 ± 0.01 cm, respectively) (Table 2). A highly significant change ($P \leq 0.01$) was detected in the antibacterial activity of treated queens against *E. coli* 2 compared with that of healthy queens, but there was no significant change compared with water-fed queens. In *K. pneumoniae*, there was a highly significant increase in the antibacterial activity of bacterially treated queens compared with that of healthy and water-fed ones. In contrast, no significant differences were observed in treated queens against *E. coli* 1 (Fig. 2).

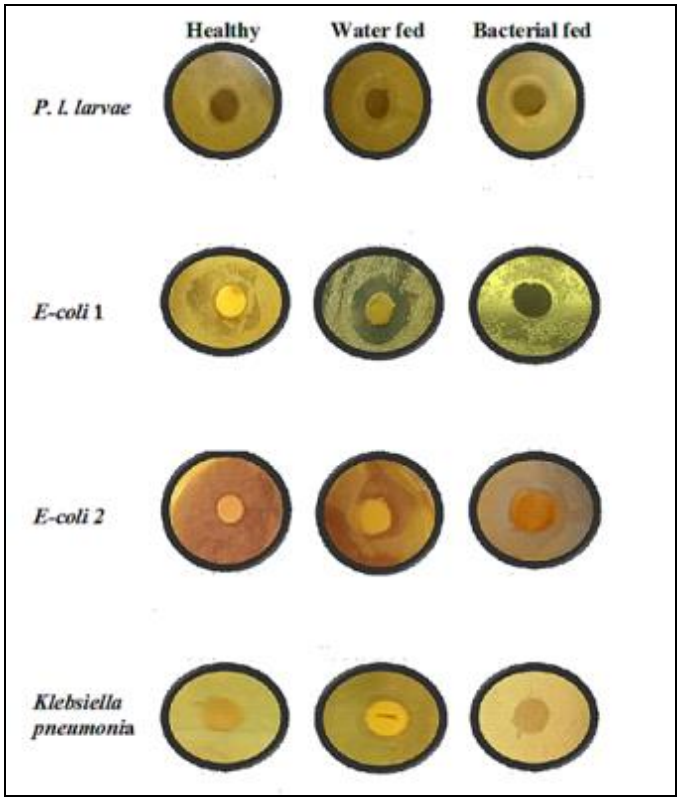

Fig. 1. Antibacterial zone activity of the body homogenate for emerging queens, namely, *A. mellifera* healthy queens (negative control), water-fed queens (positive control), and bacterially fed queens (treated) against *P. l. larvae, E. coli* 1*, E. coli* 2, and *K. pneumoniae*.

Table 2. Antibacterial effect of the body homogenate for *A. mellifera* for healthy, water-fed, and bacterially fed queens against *P. l. larvae* and multidrug-resistant bacteria *E. coli* 1*, E. coli* 2, and *K. pneumoniae*.

Bacteria	Antibacterial zone activity (cm) Mean ± SE		
	Healthy	+ve Control	Treated
Paenibacillus larvae larvae	0.82 ± 0.06	1.3 ± 0.07	0.84 ± 0.05
E. coli 1	1.3 ± 0.12	1.5 ± 0.2	1.25 ± 0.05
E. coli 2	0.45 ± 0.02	0.92 ± 0.2	0.65 ± 0.02*
Klebsiella pneumoniae	0.45 ± 0.02	0.5 ± 0.01	0.65 ± 0.01*

N=3, where three replicates were used for each treatment.
*Significant (P ≤ 0.01).

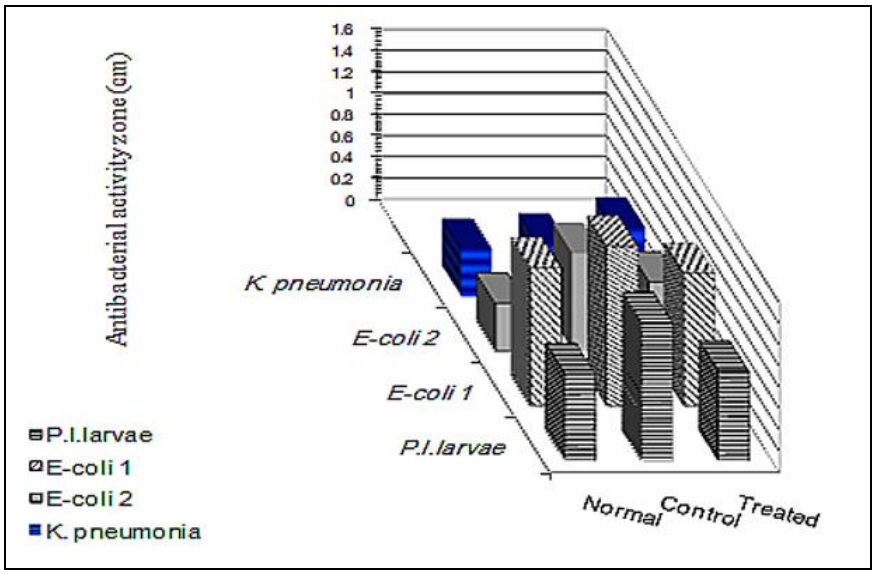

Fig. 2. Antibacterial activity (cm) of the body homogenate for *A. mellifera* queens (Normal: healthy queens; Control: water-treated queens; Treated: bacterially treated queens) against *P. l. larvae* and different multidrug-resistant bacteria: *E. coli* 1*, E. coli* 2, and *K. pneumoniae*.

Antibacterial Activity of The Ovary Extract:

The bactericidal activity of the honeybee queens' ovary extract against the bacteria *P. l. larvae*, *E. coli* 1, *E. coli* 2, and *K. pneumoniae* as determined using the agar disk diffusion technique is shown in Figure 3. The mean dimensions of the inhibition zones were 0.55 ± 0.02, 0.98 ± 0.03, and 0.92 ± 0.05 cm for healthy queens, water-fed queens, and bacterially fed queens, respectively. The ovary extract of treated queens showed a highly significant increase ($P < 0.01$) in antibacterial activity against *P. l. larvae* compared with the healthy group and no significant change compared with the water-fed group (Table 3). The mean dimensions of the inhibition zones of healthy, water-fed, and treated queens against *E. coli* 1 were (0.65 ± 0.23, 0.56 ± 0.02, and 0.85 ± 0.4 cm, respectively). In *E. coli* 2, inhibition zones were (0.7 ± 0.001, 0.6 ± 0.001, and 0.65 ± 0.3 cm) for healthy, water-fed, and treated queens, respectively. Inhibition zone dimensions of *K. pneumoniae* were (0.49 ± 0.001, 0.51 ± 0.0003, and 0.67 ± 0.0004 cm, respectively) (Table 3). A highly significant change ($P \leq 0.01$) was detected in the antibacterial activity of treated queens' ovary extract against *E. coli* 1 compared with that of the healthy group, but no significant change was detected compared with water-fed

queens. For *K. pneumoniae,* there was a highly significant increase in the antibacterial activity of bacterially treated queens compared with that of healthy and water-fed queens. In contrast, no significant differences were observed in treated queens against *E. coli* 2 compared with the levels in healthy and water-fed groups (Fig. 4).

Table 3. Antibacterial effect of the ovary homogenate for *A. mellifera* for healthy, water-fed, and bacterially fed queens against *P. l. larvae* and multidrug-resistant bacteria: *E. coli* 1*, E. coli* 2, and *K. pneumoniae.*

Bacteria	Antibacterial zone activity (cm) Mean ± SE		
	Healthy	+ve Control	Treated
Paenibacillus larvae larvae	0.55 ± 0.02	0.98 ± 0.03	0.92 ± 0.05*
E. coli 1	0.65 ± 0.23	0.65 ± 0.02	0.85 ± 0.4*
E. coli 2	0.7 ± 0.001	0.6 ± 0.001	0.65 ± 0.3
Klebsiella pneumoniae	0.49 ± 0.001	0.51 ± 0.0003	0.67 ± 0.0004*

N=3, where three replicates were used for each treatment.
*Significant ($P \leq 0.01$).

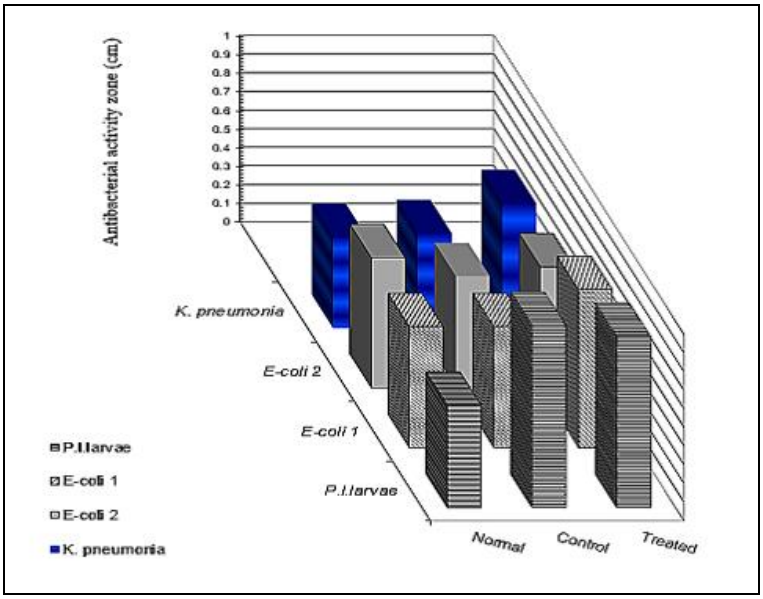

Fig. 4. Antibacterial zone activity (cm) of the ovary homogenate of *A. mellifera* queens (Normal: healthy queens; Control: water-treated queens; Treated: bacterially treated queens) against *P. l. larvae* and different multidrug-resistant bacteria: *E. coli* 1*, E. coli* 2, and *K. pneumoniae*.

DISCUSSION

Several authors identified proteins that are immune responsive (Evans, 2004; Evans and Lopez, 2004; Guidugli *et al*., 2005; Randolt *et al*., 2008), causing the production of antibacterial proteins and peptides (more than 50 factors). These proteins start to accumulate in the hemolymph within a few hours after bacterial treatment (Hultmark, 1993). Most of these peptides and proteins act by disintegrating the bacterial membrane or interfering with membrane assembly (Otvos, 2000). The interactions of plasma components and material within hemocytes produce an inducible bactericidin, which has limited specificity because it can be induced by heterologous (nonspecific) antigens (Bakula, 1971). In these experiments, immunity was not linked to any

immunoglobulin-like components in the hemolymph. Insects, like all other invertebrates, lack immunoglobulins, but they do have several components with varying degrees of specificity, such as lectins, cytokine-like molecules, and AMPs (Ottaviani, 2005). The plasma lysozyme hydrolyzes bacterial peptidoglycan and sends a signal to the fat body to begin making antibacterial proteins. These substances can directly inhibit bacterial growth (Morishima *et al.,* 1992).

AMPs are a class of peptides having antibiotic and antifungal effects. They are also known as host defense peptides and are involved in inflammation, wound healing, regulating the adaptive immune system, and homeostasis (Auvynet and Rosenstein, 2009). These peptides are evolutionarily conserved molecules that are involved in the defense of most living organisms. The biochemical properties of AMPs vary, but they typically act directly against microbes via a mechanism involving membrane disruption and pore formation, which leads to leakage of the cell contents and cell death (Lapis, 2008). They can also target intracellular components including DNA, enzymes, and even organelles (Teixeira *et al*., 2012). AMPs from the defensin family may play vital roles in orchestrating innate immune responses and contribute to the interplay between innate and adaptive immunity (Kruse and Kristensen, 2008; Gomes and Fernandes, 2010). Furthermore, abaecin is an AMP that is highly effective against hymenopteran-infecting gram-negative bacteria (Kim *et al*., 2007), such as in *A. mellifera* (Cateels *et al*., 1990), *Bombus pascuorum* (Rees *et al*., 1997), and *B. ignitus* (Choi *et al*., 2008). It is rapidly produced in the fat body after septic injury or immune challenge, and then released into the hemolymph where it acts against microorganisms (Cateels *et al*., 1990; Choi *et al*., 2008). Understanding how the insect immune system combats pathogens may aid the development of new strategies to block the transmission of disease agents (Christophides, 2005).

Multidrug-resistant pathogens have been found in large numbers all over the world. Traditional antibiotics are ineffective at treating multidrug-resistant bacteria (Farmanullah *et al*., 2020), so there is a worldwide push to find antimicrobial alternatives (Rima *et al*., 2021). AMPs have emerged as promising agents to combat antibiotic-resistant microorganisms. They are essential components of innate immunity, allowing humans to resist microbial infection. Their role has evolved from that of simple endogenous antibiotics to that of multifunctional mediators, and antibacterial activity is most likely not their only major function (Zaiou, 2007). Our study was therefore conducted to determine the antibacterial activity of the total body and ovary homogenates of treated emerging queen honeybees against multidrug-resistant bacteria. Immunization of honeybee queens was performed using a sublethal dose of the bacterium *P. l. larvae*. In insects, immune priming includes challenge with a non-pathogenic microbe or exposure to a low dose of pathogenic microbes, which gives significant protection against subsequent pathogenic infection (Patrnogic *et al.,* 2018). The results obtained here proved that healthy and bacterially treated queens attained acceptable antibacterial activity against gram-positive bacteria *P. l. larvae* and gram-negative multidrug-resistant bacteria (*E. coli* 1, *E. coli* 2, and *K. pneumoniae*); particularly significant increases of antibacterial activity against *P. l. larvae* and *E. coli* 1 were found from the queens challenged with bacteria. AMPs could have a considerable impact given the treatment failures associated with multidrug-resistant bacteria, which have become a global concern to public health (Guschin *et al*., 2015; Martin *et al*., 2015). AMPs have the potential to become a new generation of promising antimicrobial agents in future anti-infection applications (Abou Nader *et al*. 2021). Natural products remain a major source of new therapeutic compounds (Balouiri *et al*., 2016). Such products are derived from prokaryotic bacteria, eukaryotic microorganisms, plants, and diverse animals. These peptides could be applied

to produce new therapeutic agents (Diamond *et al.*, 2009) that are complementary to antibiotics because they can inhibit bacteria (Farmanullah *et al.* 2020) as well as an unusually broad range of microbes (Auvynet and Rosenstein, 2009).

Conflict of Interest: The authors declare no conflicts of interest.

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