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Research Article

Determination of Antimicrobial Activity and Total Hemocyte Count in the Larval Hemolymph of *Galleria mellonella* (L.) (Lepidoptera: Pyralidae) Following Application with *Fusarium proliferatum*

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Abstract: The impact of entomopathogenic viruses, bacteria, fungi and nematodes on the immune responses of insects has been extensively examined in model and medically important insects. However, the single time point selected in these studies presents a challenge in comprehensively understanding immune responses throughout infection in pest species. The objective of this study was to gain insight into the cellular and humoral immune responses of *Galleria mell*onella larvae, a model organism, to infection with the entomopathogenic fungus *Fusarium proliferatum* at two different time points (24h and 48h). In the antimicrobial activity tests conducted as part of the humoral immunity studies, hemolymph was induced by varying concentrations of conidial doses. After conidial dose applications, the largest zone diameters were observed against *Klebsiella pneumonia, Saccharomyces cerevisiae*, *Salmonella typhimurium* (15 mm/24h), *Proteus vulgaris* (16 mm/24h), and *Escherichia coli* (18 mm/48h). Topical application of fungal conidia to *G. mellonella* larvae in the later stages reduced the total hemocyte count in the larval hemolymph 24h and 48h after treatment. Our findings show that the immune system of *G. mellonella* responds differently to *F. proliferatum* depending on the infection timeline. Further studies on fungal regulation of the immune system could provide new pest control methods in agriculture.

Keywords: *Galleria mellonella*, *Fusarium proliferatum*, Immune responses, Antimicrobial activity, Total hemocyte count, Biological control.

1. Introduction

Entomopathogenic fungi cause disease in insects and regulate their populations (Wang et al., 2021). These fungi offer a number of benefits as an eco-friendly alternative to chemical insecticides (Chen et al., 2021). Insects have evolved defence mechanisms to combat infection. The cuticular integument is the initial physical barrier, preventing pathogens from entering the body (Leger et al., 1991). The innate responses of insects are characterised by both humoral and cellular mechanisms (Schmidt et al., 2001; Er, 2010). The introduction of a foreign particle into the hemocoel results in a series of cell-mediated responses, including alterations in hemocyte numbers, coagulation, and melanization. Additionally, humoral immunity is activated, leading to the

synthesis of antimicrobial peptides (AMPs) and lysozymes (Schmid-Hempel, 2005). The activation of enzymes and the phenoloxidase cascade, along with the production of oxygen and nitrogen intermediates, also contribute to this process (Haine et al., 2008; Ye et al., 2009; Laughton et al., 2011). The interplay between humoral and cellular immune responses consequently exerts an effect on the functions of hemocytes, which produce and release AMPs (Lavine et al., 2002). The *Galleria mellonella* (L.) (Lepidoptera: Pyralidae) is a frequently utilised model organism for the investigation of the insect immune response at the cellular and humoral levels. The quantity of hemolymph obtainable from each larva is approximately 20-50 µL, rendering it a preferred subject of study in various research areas, including host-pathogen interactions, mechanisms of fungal pathogenesis and the potential of biocontrol agents (Lionakis et al., 2011; Tsai et al., 2016). Furthermore, it has been demonstrated that the immune response of this insect is highly specific, capable of discriminating between different classes of microorganisms (Trevijano-Contador and Zaragoza, 2019).

Insect immune responses have been studied since the early 1900s, focusing on application to human infections and diseases. However, natural insect pathogens are more appropriate for stimulating insect immune responses (Zahirnia et al., 2023). *Fusarium* species that are pathogenic to insects have a wide range of hosts, including Lepidoptera, Hemiptera, Diptera, Hymenoptera and Coleoptera (Teetor-Barsch and Roberts, 1983). *Fusarium* spp. can be isolated from different insect developmental stages (Vannini et al., 2017). *Fusarium proliferatum* is an entomopathogenic fungus isolated from different insects worldwide (Al-Ani et al., 2018). In a study conducted by Tosi et al. (2015), it was isolated from the chestnut gall wasp and evaluated as a biological control agent against *Dryocosmus kuriphilus* (Hymenoptera: Cynipidae). As a result of this study, it was found to cause the death of larvae, pupae and adults of *D. kuriphilus*. Controlling insects with fungi depends on a balanced interaction between the host, pathogen and environment. Understanding which insects are susceptible or resistant to a pathogen is key (Chouvenc et al., 2009). The aim of this study is to analyse the immune response of a model organism, *G. mellonella* larvae, in the control of *F. proliferatum* conidia and to evaluate the results in terms of whether the immune response is activated (taking into account changes in cellular/total hemocyte counts (THC) and humoral/AMPs presence). The comparative results of antimicrobial activity in induced and non-induced (control) hemolymph and THC after inoculation of *G. mellonella* larvae with different concentrations of *F. proliferatum* conidia were presented with data obtained 24h and 48h after infection.

2. Materials and Methods

2.1. Rearing of *G. mellonella*

A laboratory colony of *G. mellonella* was established in the Department of Biology, Faculty of Arts and Sciences, Balikesir University (BAUN), Turkey. The laboratory conditions were maintained at 26±2 °C, with a humidity level of 65±5% and a 12:12 L:D cycle. The culture was fed using a synthetic feeding medium comprising wheat bran (500 g), honey (150 mL), glycerol (300 mL), distilled water (150 mL) and beeswax (200 g), as described by Sak (2004).

2.2. Fungus

F. proliferatum (P4-EP19) was stored in the Mushroom Herbarium of Balikesir University, Department of Biology, Microbiology Laboratory, Turkey. To prepare the conidial suspension, three-point inoculations were conducted on Malt Extract Agar (MEA, Oxoid, CM 59) in Petri (90 mm) at 28 °C for 14 days. The stock culture was preserved in MEA at -20 °C.

2.3. Preparation of conidial suspension

MEA-grown colonies, 14 days at 28 °C/dark, were used to prepare the conidial suspension. Sterile water (10 mL) containing Tween 20 (RPI, P20370-1.0) (0.01%) was added to the Petri (90 mm) in order to scrape the spores. Mycelia and agar were removed and the suspension transferred to 50 mL sterile Falcon tubes. A Thoma slide was used to count conidia and suspensions were prepared at concentrations of $1x10^9$ cfu/mL (Fancelli et al., 2013).

2.4. Hemolymph collection

Conidial suspensions (1x10⁵-1x10⁹ cfu/mL) were freshly prepared for *G. mellonella* larvae (250±25 mg). The doses were immediately applied to the larvae by topical application to the dorsal thorax with a micropipette without delay. The treated individuals were placed in 60x15 mm plastic Petri dishes and kept in incubators with a 12:12 L:D photoperiod at a temperature of 26±2 °C and humidity of 65±5% until the end of the application hours. Hemolymph samples were collected from control and experimental groups after 24h and 48h. Larvae were chilled at 4 °C for 15 minutes before collection. To avoid melanisation, hemolymph samples were transferred to sterile and refrigerated Eppendorf containing 1-phenyl-2-thiourea (1 mg/ Merck, 179817) (Radwan et al., 2022; Güner et al., 2023; Scieuzo et al., 2023). The hemolymph samples obtained were stored at -20 °C.

2.5. Antimicrobial activity assay

2.5.1. Microorganisms

For antimicrobial activity, Gram-positive bacteria *Staphylococcus aureus* (SA, ATCC 6538P), *Bacillus cereus* (BC, CCM 99), Methicillin-resistant *Staphylococcus aureus* (MRSA, ATCC 33592), *Streptococcus agalactiae* (SAG, ATCC 23956) and Gram-negative bacteria *Klebsiella pneumoniae* (KP, CCM 26 2318), *Escherichia coli* (EC, ATCC 11230), *Proteus vulgaris* (PV, ATCC 6897), *Serratia marcescens* (SM, ATCC 43861), and *Salmonella typhimurium* (ST, ATCC 14028) were used. In addition, two fungal pathogens, *Candida albicans* (CA, ATCC10239) and *Saccharomyces cerevisiae* (SC, ATCC 4098), were also utilized.

2.5.2. Disc diffusion method

To determine the antimicrobial activity induced by different doses of *F. proliferatum* in larval hemolymph against different bacteria, the disc diffusion method was performed using Mueller Hinton Agar (MHA, BD211438) (Saad et al., 2021). The inoculum suspensions were prepared in accordance with the 0.5 McFarland standard, utilising 24h fresh cultures of the microorganisms under investigation. The inoculum suspension was prepared using microorganisms in a solution of 0.85% w/v NaCl (El-Saadony et al., 2021). The control groups were established using tobramycin (Oxoid, CT0056B /TOB-10 µg) and sulphamethoxazole trimethoprim (Oxoid, CT0052B/SXT-25 µg) antibiotic discs, PBS (Phosphate Buffered Salt, Sigma, Aldrich/P4417), non-induced and PBS-induced hemolymph samples. A total of 10 µL of hemolymph was collected from both the experimental and control groups and added to sterile blank paper discs (Oxoid, CT0998B/6mm). Following the preparation of the suspension (500 µL), was distributed across the surface of the Petri (90 mm). The prepared discs were then placed on the surface of the Petri with the assistance of forceps. The Petri of all experimental groups were incubated at 37 ± 0.1 °C for a period of 24 h. Inhibition zones were measured in millimetres (mm) at the end of the incubation period. The results, including zone diameter and disc diameter, were compared with standard antibiotic discs and controls (Marshall and Arenas, 2003) to validate the experiment and provide a benchmark for the results. Zone diameter and disc diameter were measured together to calculate zone width.

2.6. Determination of total hemocyte count (THC)

For studies to determine the effects of conidial suspensions at different concentrations on the THC of *G. mellonella*, fresh conidial suspensions were prepared at doses of 1x10⁵-1x10⁹ cfu/mL. The doses were immediately applied to the larvae using the topical application method. A micropipette was used to apply the dorsal side of the thorax. The treated individuals were placed in plastic Petri (60 mm) and kept at 26±2 °C, 65±5% humidity and a 12:12 L:D photoperiod until the end of the application times. Hemolymph was collected from larvae 24h and 48h after dose application (Er, 2011; Güner, 2024). Control groups consisted of non-induced individuals and groups treated with PBS. Each dose and control group consisted of a total of 5 larvae. To determine for THC, the larvae were punctured with a fine needle (lancet) from their first hind legs, and hemolymph (4 µL) was collected from each larva using a microcapillary tube (Hirschmann, Z611239). Each hemolymph sample was transferred to Eppendorf tubes containing an anticoagulant solution (0.098 M NaOH, 0.186 M NaCl, 0.017 M Na_2EDTA and 0.041 M citric acid, pH = 4.5). The cell suspension was diluted to a ratio of 1:10. The cell suspension (10 µL) was withdrawn with a micropipette and loaded into a

Neubauer hemocytometer (Improved Neubauer Hemocytometer; Superior, Germany). The THC per milliliter was calculated using the following formula, as proposed by Er (2011), based on the hemocytes counted under the microscope (Olympus BX51).
Cell count / mL = number of cells counted in the large square x dilution factor x 10^4

2.7. Statistics

The data obtained from applying of *F. proliferatum* as conidial suspensions at different doses to *G. mellonella* were individually subjected to the One-Way Analysis of Variance Test (ANOVA). The Tukey HSD Test was applied to control the significance of the difference between means (SPSS 18.0, Chicago, IL). A confidence limit of 0.05 was used as the reference in the evaluations.

3. Results

The quantities of hemolymph obtained from the control and experimental groups at two different time points were presented in Table 1.

Table 1. Hemolymph amounts.

Groups	Collected hemolymph (µL)
Control (normal)	605 µL
PBS-induced Control (24h)	485 µL
PBS-induced Control (48h)	450 µL
10^9 cfu/mL-(24h)	585,5µL
10^9 cfu/mL-(48h)	555 µL
10^8 cfu/mL-(24h)	590 µL
10^8 cfu/mL-(48h)	485 µL
10^7 cfu/mL-(24h)	550 µL
10^7 cfu/mL-(48h)	445 µL
10^6 cfu/mL-(24h)	555,5 µL
10^6 cfu/mL-(48h)	410 µL
10^5 cfu/mL-(24h)	480 µL
10^5 cfu/mL-(48h)	575 µL

The objective of this study was to evaluate the antimicrobial activity levels of hemolymph collected at two distinct time points (24h and 48h) from *G. mellonella* larvae infected with *F. proliferatum*. This assessment was conducted using the disc diffusion method. As illustrated in Figure 1, the infection of larvae with *F. proliferatum* results in the emergence of antimicrobial activity within the hemolymph, directed against the tested bacteria and yeasts. The results for the non-induced and PBS-induced control group were presented together with those for the PBS and standard antibiotics (TOB-10 µg and SXT-25 µg) (Table 2). No antimicrobial activity was detected on disks prepared with PBS, while the largest inhibition zone diameter (43 mm) was observed with SXT against *S. aureus* and *K. pneumoniae*. The antimicrobial activity results of hemolymph induced with conidial doses were provided in Table 3. The results indicate that antimicrobial activity was more strongly induced in hemolymph collected at the end of 24h, whereas antimicrobial activity began to decrease and eventually disappeared in hemolymph collected after 48h.

The THC in control larvae were 46.82 and 39.90 x 10^6 cells/mL at 24h and 48h periods, respectively. In the experiments, the hemocyte count of larvae showed significant differences between experimental and control groups at the end of both 24h ($F=7.519$; $df=6, 28$; $p=0.000$) and 48h ($F=20.434$; $df=6, 28$; $p=0.000$) (Table 4). As indicated in Table 4, there was a notable decline in THC levels following a 24-hour period, particularly in larvae administered with 10^9 cfu/mL. Furthermore, at the conclusion of the 48-hour period, THC exhibited a reduction in all groups, with the exception of the control groups.

Table 2. Anti-microbial activity of control groups.

Microorganisms	Control Group					
	H	PBS-induced H		PBS	TOB	SXT
		24-h	48-h			
E. coli	12	11	11	-	10	20
S. aureus	10	10	9	-	29	43
B. cereus	11	10	9	-	15	-
P. vulgaris	10	12	12	-	21	42
K. pnemoniae	10	9	10	-	33	43
C. albicans	11	10	11	-	22	41
S. cerevisiae	9	9	11	-	11	29
MRSA	7	8	7	-	30	40
S. typhimurium	12	10	11	-	30	40
S. marcescens	13	10	11	-	27	40
S. agalactiae	10	8	9	-	22	41

*H: Hemolymph (normal-non induced); PBS induced H: PBS induced Hemolymph; (-) not detected.

Table 3. Anti-bacterial activity of experiment group.

	Experiment Group									
	10^5 cfu/mL		10^6 cfu/mL		10^7 cfu/mL		10^8 cfu/mL		10^9 cfu/mL	
Microorganisms	24-h	48-h	24-h	48-h	24-h	48-h	24-h	48-h	24-h	48-h
E. coli	-	-	-	-	11	13	11	14	13	18
S. aureus	7	7	8	7	8	8	9	11	11	12
B. cereus	-	-	-	-	-	-	9	-	10	-
P. vulgaris	10	10	12	11	13	13	14	13	16	15
K. pnemoniae	-	-	8	-	9	-	13	8	15	8
C. albicans	7	-	9	-	10	-	11	11	13	16
S. cerevisiae	7	8	9	9	11	9	13	10	15	13
MRSA	7	-	8	-	8	-	9	-	9	-
S. typhimurium	10	-	11	-	11	7	12	9	15	11
S. marcescens	7	7	7	7	9	8	11	8	13	15
S. agalactiae	7	-	8	-	8	-	9	-	11	-

*(-)was not detected.

Table 4. Effect of *F. proliferatum* on the THC (x10^6 cells/mL) of *G. mellonella.*

	THC (x10^6 cell/mL) ($\bar{x}$ ±SH*) Time**	
	24-h	48-h
Control	46,82±6.29a	39,90±1.63a
PBS induced	38,79±3.76a	39,08±0.57a
10^5 cfu/mL	34,10±1.20a	30,20±0.57b
10^6 cfu/mL	41,26±1.63a	33,46±1.44b
10^7 cfu/mL	39,88±0.99a	33,22±0.43b
10^8 cfu/mL	37,50±1.86a	32,98±1.96b
10^9 cfu/mL	20,51±0.79b	23,16±1.16c

*Each value represents the mean THC of five larvae. **In the same column (a-c), the differences between values sharing the same letter are statistically insignificant (P>0.05).

4. Discussion

The determination of the genotoxic, physiological and biochemical effects of infections is considered to be a practical step in the understanding of the physiological defence characteristics of insects (Black et al., 2022). The immune system is an important indicator of their susceptibility to various types of contamination by foreign pathogens, including fungal and bacterial spores, toxins, diapause, molting, starvation stress, environmental conditions and diet alterations (Mowlds et al., 2008). The objective of the experiments described here were designed to investigate the effects of *F. proliferatum* on the immune responses of a model insect and to determine whether fungal application makes the insect more susceptible to subsequent pathogen efficacy. In order to develop effective strategies for biological control studies with fungi, it is essential to gain insight into the mechanisms by which insects resist fungal spores or their by-products and to determine the biological responses in susceptible hosts. The findings of this study contribute to our understanding of the impact of *F. proliferatum* on the cellular and humoral immune responses of *G. mellonella* larvae.

The first line of defence against invading pathogens or parasites is provided by hemocytic immunity (Lavine and Strand, 2002). It can be reasonably assumed that the process of hemocyte immunity is initiated within a few seconds of the detection of infection. In contrast, the AMPs involved in humoral immunity have been observed to take between six and 12 hours to appear in the hemolymph of infected insects (Stanley and Kim, 2014). It was documented (Zhang et al., 2009) that the injection of bacteria or fungi into the hemocoel of an insect leads to an increase in the synthesis of peptides and proteins secreted into the hemolymph. In this study, the antimicrobial activity of *G. mellonella* larval hemolymph was evaluated at 24 and 48 h post-application of *F. proliferatum* conidia using the disc diffusion method. The increase in the antimicrobial activity of insect hemolymph can be attributed to an elevation in the production of AMPs as a consequence of microbial exposure (Uvell and Engström, 2007). The results obtained so far indicate that *F. proliferatum* induces antimicrobial activity in hemolymph collected 24h after application of high doses of conidia (10^8 and 10^9 cfu/mL). Korner and Schmid-Hempel (2004) reported that the antimicrobial activity of hemolymph in *Zophobas atratus* (Coleoptera: Tenebrionidae) and *Bombus terrestris* (Hymenoptera: Apidae) reached a maximum 24 to 48 hours after microbial application. Güner et al. (2023) showed a similarity with our research by stating that the antimicrobial activity of the hemolymph of *Ephestia kuehniella* (Lepidoptera: Pyralidae) larvae induced by *Penicillium mallochii* increased significantly after 24h compared to the control groups. Of all the results, the largest zone diameter (18 mm/48h) was observed against the gram-negative bacteria *E. coli.* This result was obtained from the hemolymph collected 48h after the application of the highest dose of the fungus. The results of the antimicrobial activity tests demonstrated that the hemolymph produced as a consequence of the conidial application was more efficacious in combating Gram (-) bacteria. The current study demonstrated that the hemolymph induced by *F. proliferatum* conferred susceptibility to *E. coli, K. pneumonia, S. typhimurium*, *S. marcescens*, and *P. vulgaris*. According to the results of the antimicrobial activity test, it was found that the hemolymph of normal insects had a low level of antimicrobial activity against the test microorganism. In insects that are not infected, the expression of antimicrobial peptides (AMPs) can be induced by changes in

metabolism, stress factors and the process of ageing (Bland, 2023). In vivo, AMPs are secreted by fat bodies and hemocytes in the absence of any external stimuli, thereby initiating an immune response. The function of immune cells in intact larvae differs depending on whether the study is conducted in vivo or in vitro. Furthermore, the AMPs titer in the plasma of these larvae is relatively low (Yakovlev, 2011). While the fat body does not produce AMPs under normal conditions, damage to the skin can result in a significant increase in the antimicrobial activity of the fat body and a widespread release of AMPs (Yakovlev et al., 2017).

The present study offers evidence of the interaction between *F. proliferatum* and the cellular immune system of *G. mellonella*. It was demonstrated that hemocytes, which are integral to the insect immune system, exhibited a reduction in THC levels in the hemolymph of last-stage *G. mellonella* larvae following the topical application of fungal conidia. This reduction was observed at both 24 and 48h post-application. This finding is consistent with the study by Sapna et al. (2015), which showed an initial increase in THC in houseflies infected with *Beauveria bassiana* (6-9h), followed by a decrease with prolonged infection duration (9-12h). Similar to our results, a significant decrease in hemocyte counts after fungal infection with *Conidiobolus coronatus* was observed in *G. mellonella* by Bogus et al. (2017). In another study, a significant reduction in various hemocyte counts was observed in *Bombyx mori* (Lepidoptera: Bombycidae) and *Spodoptera exigua* (Lepidoptera: Noctuidae) larvae infected with *B. bassiana* (Rajitha et al., 2013). This reduction has been linked to encapsulation as an immune response to fungal infection (Andrade et al., 1984; Rivers et al., 2002). Studies on some Lepidoptera species have shown changes in hemocyte numbers in response to injury, bacterial, fungal and viral infections and various stresses (Mowlds et al., 2008; Kim and Kim, 2010). The host THC increases (Sewify and Hashem, 2001) or decreases (Avulova and Rosengaus, 2011) following a fungal infection. Some studies have suggested that the THC in insect hemolymph may increase in response to pathogen infection due to induced ematopoiesis and hemocyte mitosis (Kumar et al., 2011). A decrease in THC can lead to cellular immunosuppression, which favours the infecting fungus (Zibaee et al., 2011; Yu et al., 2016).

It is hypothesised that *G. mellonella*, in their attempts to cope with increasing conidia doses, will eventually experience a weakened larval defence due to the continued stress conditions affecting repair mechanisms. The study of insect hemocytes has concentrated on a number of key areas, including the effects of starvation, infection and injury (Sewify and Hashem, 2001), the processes of mitotic division and the alterations in hemocyte and hemocyte number (Yamashita and Iwabuchi, 2001), mitotic division at different developmental stages, and apoptosis during hemocyte function (Le et al., 2003). Despite the existence of studies examining the impact of diverse fungal species on THC in insects, there is a notable absence of research investigating the relationship between *Fusarium* species and THC in *G. mellonella* larvae.

5. Conclusion

The data indicate that the immune system of *G. mellonella* exhibits a differential response to the fungal pathogen *F. proliferatum* at varying time points. Further research would be enhanced by the utilisation of physiological and transcriptional analyses, with the objective of enhancing comprehension of the insect's response to fungal pathogens. This may facilitate the development of novel pest control methodologies within agricultural ecosystems.

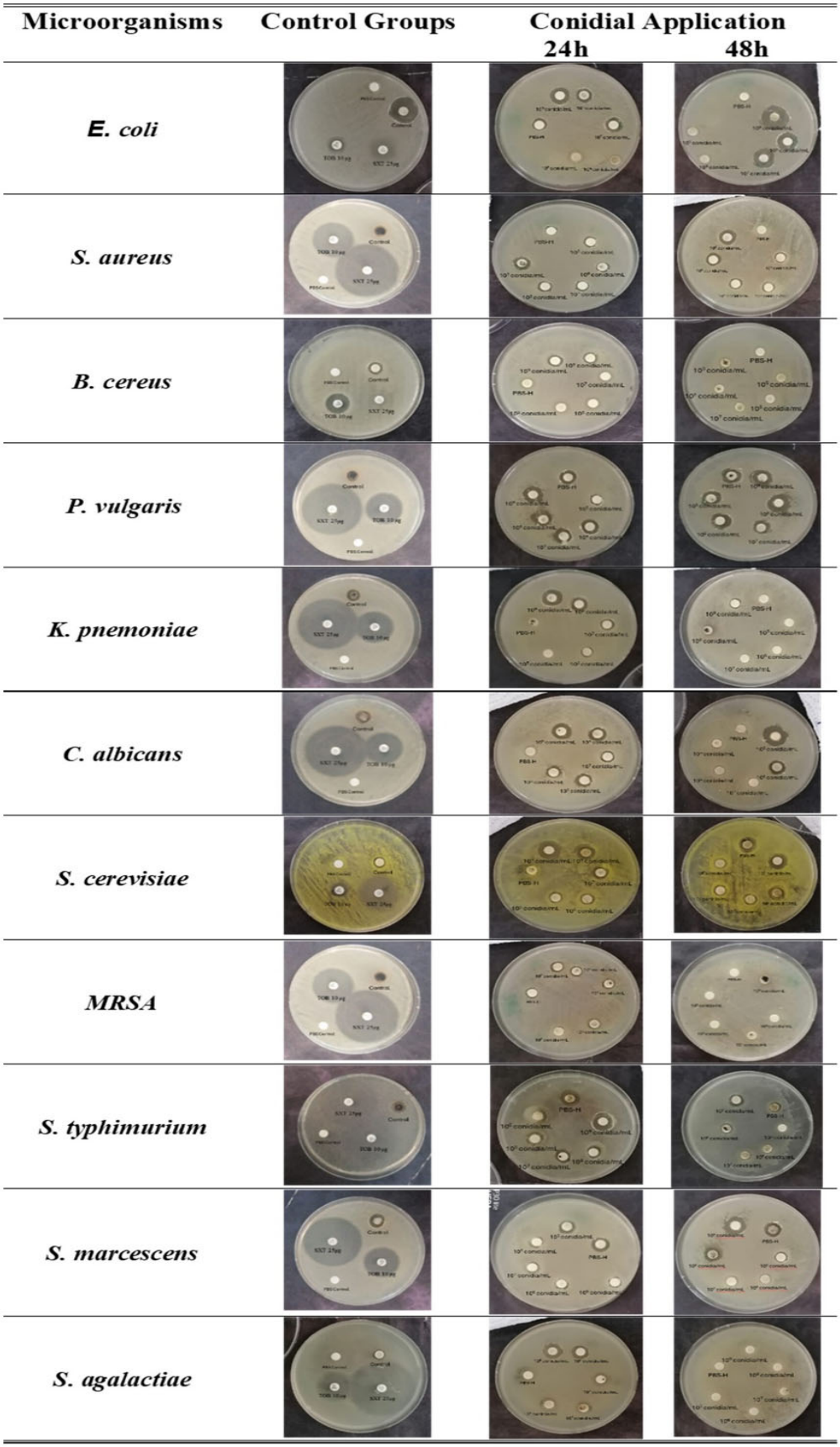

Figure 1. Photographs of the disk diffusion test results of the control and experimental groups.

Conflicts of Interests

The authors declare that they have no confict of interest.

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Statement contribution of the authors

All the authors substantially contributed to the conception and design of the manuscript. Data curation and analysis were maintained by PG, TA, AE, SMM. Writing the entire manuscript was done by PG. All authors have read, revised, and approved the manuscript.

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