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Functional characterization of outer membrane proteins (OMPs) in *Xenorhabdus nematophila* and *Photorhabdus luminescens* through insect immune defense reactions

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Summary

Xenorhabdus nematophila and *Photorhabdus luminescens* are entomopathogenic symbionts that produce several toxic proteins that can interfere with the immune system of insects. Here, we showed that outer membrane proteins (OMPs) could be involved as virulence factors during bacterial symbiont pathogenesis. Purified OMPs from bacterial culture were injected fifth instar larvae of *Spodoptera exigua* Hübner. Larvae were surveyed for fluctuations in total haemocyte counts (THC), granulocyte percentage (cellular defence), protease, phospholipase A2 (PLA_2), and phenoloxidase (PO) activities (humoral defence) at specific time intervals. Changes in the expression of the three antimicrobial

peptides (AMPs), cecropin, attacin, and spodoptericin, were also measured. Larvae treated with both types of OMPs had more haemocytes than did the negative controls. OMPs of *X. nematophila* caused more haemocyte destruction than did the OMPs of *P. luminescens*. The OMPs of both bacterial species initially activated insect defensive enzymes post-injection, their activating fluctuated in different ways. Attacin, cecropin and spodoptericin were up-regulated by OMP injections more than in normal larvae. The expression of these three AMPs was maximal at four hpi with *P. luminescens* OMPs treatment. Expression of the three AMPs in *X. nematophila* treatment was irregular and lower than in the *P. luminescens* OMPs treatment. These findings provide insights into the role of OMPs of entomopathogenic nematode bacterial symbionts in countering the physiological defenses of insects.

Keywords: Antimicrobial peptides, Cellular defense, Insect pathology, Phenoloxidae, Phospholipase A2, Protease

1. Introduction

Xenorhabdus nematophila and *Photorhabdus luminescens* are Gram-negative bacteria (Family Enterobacteriaceae) symbiotically associated with the entomopathogenic nematodes (EPNs), *Steinernema carpocapsae* Weiser and *Heterorhabditis bacteriophora* Poinar, respectively (1). The EPN infective juvenile stage (IJ) harbors the bacteria in their intestine releasing them into the haemocoel of the host causing insect death within 24-48 hours post infection (2). Several factors characterized in *X. nematophila* and *P. luminescens* (Txp40 toxin, Tc toxin, 17-kDa pilin protein subunit) have important role in the pathogenicity of EPNs (3; 4), the bacteria contributing to killing the host by overcoming immune activities (5).

The pathogenicity of some Gram-negative bacteria depends on their ability to secrete virulence factors into the mammalian host by releasing outer membrane vesicles (OMVs) (6). Some OMVs

virulence factors include phospholipase C, proteases, elastases, hemolysins (6), phospholipids, lipopolysaccharides (LPS) [also known as endotoxins (2)], alkaline protease, and in addition to vesicles, outer membrane contains integrated membrane proteins (OMPs). In pathogenic bacteria, some of these OMPs identified as virulence factors and served for escaping of host defence mechanisms (7). Previously, a few OMPs in *Xenorhabdus* and *Photorhabdus* were identified which including *skp* in *P. temperata* and *ompF*, *OpnS and OpnP* in *X. koppenhoeferi* (8; 9). Major defensive factors of insect immune systems are the interactive cellular (haemocyte) and humoral elements. In *S. exigua* the major haemocyte types reacting against bacteria include the granulocytes and plasmatocytes (10) which respond to antigens by phagocytosis and nodulation (11). Humoral factors in this insect species include the synthesis of antimicrobial peptides (AMPs) *e.g*. cecropins, attacins, the pattern recognition protein lysozyme (12) and active prophenoloxidase cascade and phospholipase A_2 (PLA_2) (11).

Due to limited information about OMPs of *X. nematophila* and *P. luminescens*, the current study was designed to survey the effects of the OMPs on aspects of cellular and humoral defensive enzymes in the haemolymph of *S. exigua* larvae. We surveyed total haemocyte counts (THC), differential haemocyte counts (DHC), and protease, phospholipase A_2 (PLA_2), and phenoloxidase (PO) activity, as well as the expression patterns of cecropin, attacin and spodoptericin in response to expose with purified OMPs of *X. nematophila* and *P. luminescens*.

2. Material and methods

2.1. Insect culture

Different larval stages of *Spodoptera exigua* collected from the sugar beet fields at [Mashhad, Razavi Khorasan province (36º:29′ N, 59º:60′ E), Northeastern Iran] were reared at 25±1°C, under a 16:8 (L:D) h photoperiod, at R.H. 60±5% in the laboratory on sugar beet leaves. Moths were fed with

20% honey solution. Then, the eggs were collected daily. One-day-old larvae were fed with fresh sugar beet leave and the fifth instar larvae with 0.78 ± 0.026 mg weight were used for the experiments.

2.2. Purification of OMPs

2.2.1. Bacteria growth

Photorhabdus luminescens and *X. nematophila* were isolated from the *H. bacteriophora* and *X. nematophila* (e~nema company) respectively. Then cultured on NBTA medium containing nutrient agar, triphenyl tetrazolium chloride (0.004% wt/vol), and bromothymol blue (0.0025% wt/vol). A 48 h old colony, was transferred into the 100 ml nutrient broth (NB) medium in a 500 ml Erlenmeyer and incubated (at 28±1°C, 120 rpm). After 24 h the bacteria were cultured again in 1 liter volume nutrient broth (each 100 ml nutrient broth (NB) medium in a 500 ml Erlenmeyer) and shaken of 100 rpm on a horizontal shaker.

2.2.2. Preparation and fractionation of OMPs from symbiotic bacteria

The OMPs were prepared from the culture supernatant as described by Korhonen *et al*. (13) with modifications. Briefly, after growth for 48 h in NB, the cells were collected by centrifugation (15 min at 4000×g). The pellets were suspended in TEB buffer (1 mM benzamidine, 1 mM EDTA pH, 8, 5 mM Tris-HCl pH, 8) and homogenized by micropestle. Cell debris was collected by centrifugation (5 min at 2000×g) and the supernatant collected. In the collected supernatant the OMPs were precipitated by adding crystalline ammonium sulfate to 50% saturation followed with incubation at 4ºC overnight. The precipitate was collected by centrifugation (1 hour and 10000×g), dissolved in 1 ml of 5 mM Tris buffer, and dialyzed for 48 h against 5 mM Tris buffer. Sodium deoxycholate (0.5% w/v) was added to the suspension which was then dialyzed against 5 mM Tris buffer containing sodium deoxycholate (0.5% w/v) for 48 h. The suspension was centrifuged for 10 min at 10000×g. The pellet was contained

DOC-insoluble material (outer membrane proteins). The wet weights of OMPs were measured and 50 mg of OMPs dissolved in 1 ml of TENS buffer (50 mM Tris-HCl [pH 7.2], 400 mM NaCl, 5 mM EDTA, 1% sodium dodecyl sulfate). The concentration OMPs were determined and their molecular weight estimated by SDS page.

2.3. Injection of pathogens OMPs

The fifth instar larvae of *S. exigua* were injected with 5 µl OMPs (50 mg/ml) in TENS buffer separately by an insulin syringe (30 G, B. Braun; Germany). Negative control larvae received 5 µl TENS buffer. In the gene expression experiment, there was a treatment without injection was considered as the normal sample. The larval groups were kept at room temperature and fed with fresh leaves. The different aspects of immune defense were surveyed at 0.5, 2, 4, 8, 12, and 16-hour post-injection (hpi).

2.4. Total haemocyte counts and differential haemocyte counts (THC and DHC)

For THC assay, the surface body of injected larvae disinfected with ethanol. Five 5 µl of hemolymph were collected by cutting the prothoracic leg. Then, haemocytes counted using Neubauer hemocytometer (Marienfeld, Germany). Haemocyte numbers were calculated based on Jones´s formula (14). For DHC, haemolymph (10 µl) was smeared on the glass microscope slide. The cells fixed with acetic acid: methanol (1:3 v:v) for 5 min (15) and stained with 10% (V/V) Giemsa (16). One hundred haemocytes were counted randomly and haemocyte types were recorded as a percentage of total cells. Type of haemocytes was determined according to Ribeiro & Brehelin (17).

2.5. Protease assay

For total protease activities of *S. exigua* larvae, azocasein (Sigma) was used as a substrate and the absorbance determined at 450 nm on a microplate reader (Stat Fax 3200®; Awareness Technology Inc., Florida, USA). The activity of protease was expressed as µmol dye/min/mg protein using the extinction coefficient of the chromogenic azo group produced by the cleavage of casein (18).

2.6. Phospholipase A_2 (PLA_2) assay

The PLA_2 was assayed using a modification of Radvanyi *et al*. (19). Pyrene-labeled phospholipid (Sigma) was used as a substrate. The PLA_2 activity was calculated by spectrofluotometry (Cecil CE9500) and the fluorescence intensity recorded using excitation and emission wavelengths of 345 and 398 nm, respectively.

2.7. Phenoloxidase (PO) assay

For phenoloxidase activity, L-dihydroxyphenylalanine (L-Dopa, Sigma) was used as a substrate. Hemolymph was centrifuged (2000×g, 4ºC 1 min) and the supernatant plasma used as the enzyme source. One hundred µl L-Dopa (60 mM), 90 µl phosphate buffer (pH 8.6), and 10 µl supernatant were added to microplate wells. An increase in absorbance was recorded every 30 seconds over 5 min at 492 nm using a Stat Fax 3200 Microplate Reader. One unit of PO activity was defined as the amount of enzyme that oxidizes 1 mol of L-Dopa per min per mg total plasma protein at room temperature (25±2ºC). Total protein concentration was estimated according to the Bradford method (20). Different concentration of BSA used as standard curve (21).

2.8. Gene expression

2.8.1. RNA extraction and cDNA synthesis

Total RNA was extracted from haemolymph of the larvae at 2, 4, 8, and 16-hours post injection using RNA extraction kit (Pars tous-Iran: catalogue number A101231 following the manufacturer's instructions) then were treated with DNase I (Sina colon: catalogue number MO5401) according to the manufacturer's instructions. The first-standard cDNA was synthesized with 1 µg of total RNA, oligo-dt primers and reverse transcriptase according to the manufacturer's protocol.

2.8.2. Design and synthesis of primers

For AMPs expression of the target genes, attacin, cercopin, and spodoptericin a set of primers were designed and used. Elongation factor 2 (EF2) gene was the reference gene used for normalization (22).

2.8.3. Quantitative PCR (qPCR)

qRT-PCR reactions were carried out in Optical 8-Cap Strips (BIORAD) on a BIORAD machine model CFX96 using the 2X SYBR Green master mix. The total reaction volume (20 µl) contained 10 µl of SYBR Green, 0.2 µl of each of the forward and reverse primer, and 2 µl of cDNA (1 µg concentration). The PCR conditions consisted of 95°C for 10 min; 40 cycles of 95°C for 15 s, 65°C for 30 s and 72°C for 30 s, followed by a melt curve analysis at 95°C for 10s. Subsequently a temperature transition rate of 0.5 °C/s was performed from 50 to 95°C. The threshold cycle (CT) values were determined by CFX96 software. The relative expression ratios of the target gene in treated and untreated control groups were calculated using a $2^{\wedge-\Delta\Delta CT}$ method (23). All assays were performed on two independent replicates.

2.9. Statistical analysis

The data were analyzed using two-way ANOVA [(SAS Institute, (24)]. Here OMPs and time intervals were two main effects. The effect of either factor alone and interactive effect (OMPs types × times interval) were calculated. When a significant result for the ANOVA was obtained ($p < 0.05$), a slicing test was used to measure the significant difference between means. Prior to ANOVA analysis, data were assessed for the assumption of ANOVA normality and homogeneity of variance [(SAS Institute, (24)]. All graphic data indicate the mean ± standard error (SE) of the mean, in each treatment. The experiments were done at least two times with four insects for each experiment.

3. Results

3.1. Outer membrane protein profiles

The OMPs profiles of *X. nematophila* and *P. luminescens* are different (Fig. 1). A protein with molecular mass of ~35-40 kDa was sharper than other proteins. The protein concentration in *X. nematophila* and *P. luminescens* was 1.075 and 1.554 mg/ml respectively. Also, the 260/280 ratio in *X. nematophila* and *P. luminescens* was 1.12 and 1.07 respectively.

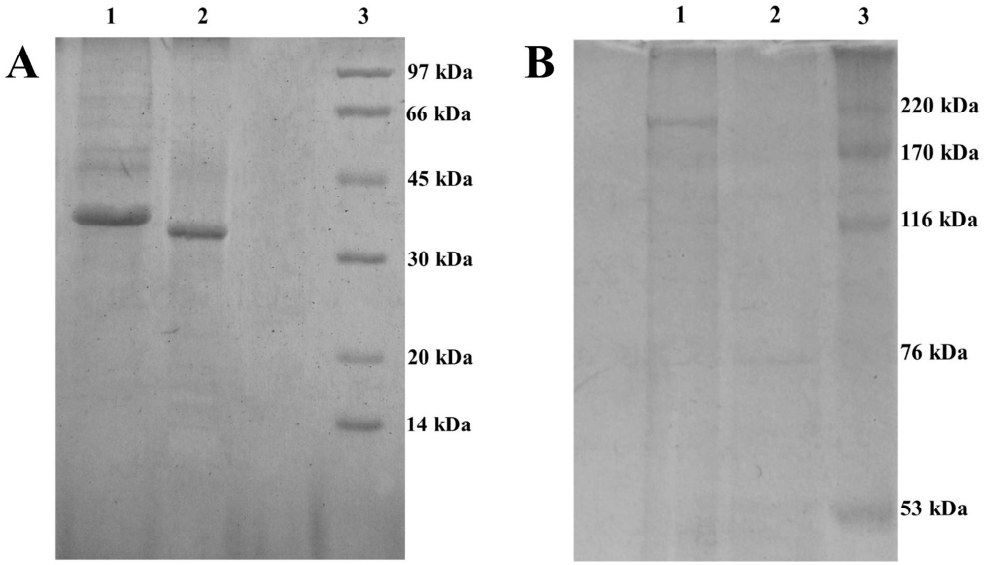

Figure 1. SDS-polyacrylamide gels showing the outer membrane proteins, 1) *Xenorhabdus nematophila*, 2) *Photorhabdus luminescens*, 3) Standard. A) The molecular weight 14-97 kDa, B) The molecular weight 53-220 kDa.

3.2. Total haemocyte count

There are differences in effects on THC depending on the OMP type and times ($F_{10,126} = 3.37$, $P < 0.05$). The OMPs ($F_{2,126} = 24.61$, $P < 0.05$) and time post-injection ($F_{5,126} = 5.67$, $P < 0.05$) significantly affected in THC values (Fig. 2A). In larvae with *P. luminescens* OMPs the haemocytes levels were more than those of *X. nematophila* OMPs during the incubation time. Also, the rate of decline in haemocyte counts in larvae with *X. nematophila* OMPs was faster than those with *P. luminescen* OMPs. In comparison with the control treatment constant THC values of the THC level in larvae with *P. luminescens* OMPs increased to maximum level at 2 hpi (2 h: $F_{1,14} = 22.11$, $P < 0.05$) then decreased from 4-16 hpi (4 h: $F_{1,14} = 7.81$, $P < 0.05$; 8 h: $F_{1,14} = 34.71$, $P < 0.05$; 12 h: $F_{1,14} = 17.53$, $P < 0.05$; 16 h: $F_{1,14} = 3.89$, $P < 0.05$). With the exception of 0.5 hpi, *P. luminescens* OMPs elevated THC above the control counts. *X. nematophila* OMPs elevated THC levels to a maximum count by 4 hpi (4 h: $F_{1,14} = 16.91$, $P < 0.05$) more slowly than did *P. luminescens* OMPs, the levels then decreased. There was a significant difference between *X. nematophila* OMPs and control treatment in THC density by 0.5 and 4 hpi (0.5 h: $F_{1,14} = 2.2$, $P < 0.05$; 2 h: $F_{1,14} = 16.46$, $P < 0.05$; 4 h: $F_{1,14} = 16.91$, $P < 0.05$). While, in larvae with *P. luminescens* OMPs a significant difference was observed compared with the negative control occurred from 2 to 16 hpi (2 h: $F_{1,14} = 22.11$, $P < 0.05$; 4 h: $F_{1,14} = 7.81$, $P < 0.05$; 8 h: $F_{1,14} = 34.71$, $P < 0.05$; 12 h: $F_{1,14} = 17.53$, $P < 0.05$; 16 h: $F_{1,14} = 3.89$, $P < 0.05$) (Figure 2A).

3.3. Granulocyte counts

There was a significant interactive effect between OMPs of both bacterial species over time (OMPs × times) ($F_{10,126} = 2.76$, $P < 0.05$). Although, the granulocyte percentage between OMPs types was not significantly different ($F_{2,126} = 1.26$, $P > 0.05$), analysis of granulocytes data over time intervals ($F_{3,126} = 148.46$, $P < 0.05$) indicated there was a significant difference among specific times.

In larvae with *P. luminescens* OMPs, the granulocyte percentages from 4 to 16 hpi were less than the negative control values (4 h: $F_{1,14} = 4.87$, $P < 0.05$; 8 h: $F_{1,14} = 3.09$, $P < 0.05$; 12 h: $F_{1,14} = 1.37$, $P > 0.05$; 16 h: $F_{1,14} = 0.48$, $P > 0.05$). The percentage of granulocytes in larvae with OMPs of *X. nematophila* was always less than the negative control. However, the granulocyte percentage increased to a maximum plateau density by 4 hpi (0.5 h: $F_{1,14} = 0.01$, $P > 0.05$; 2 h $F_{1,14} = 2.66$, $P > 0.05$; 4 h: $F_{1,14} = 1.57$, $P > 0.05$). There was a significant difference in the number of granulocyte by 0.5 hpi after injection OMPs of *X. nematophila* and *P. luminescens* (0.5 h: $F_{1,14} = 15.85$, $P < 0.05$; 2 h: $F_{1,14} = 3.77.1$, $P > 0.05$; 4 h: $F_{1,14} = 0.06$, $P > 0.05$; 8 h: $F_{1,14} = 0.95$, $P > 0.05$; 12 h: $F_{1,14} = 0.02$, $P > 0.05$; 16 h: $F_{1,14} = 3.27$, $P > 0.05$) (Figure 2B).

The changes in levels of other haemocytes including plasmatocytes, spherulocytes, and oenocytoides were calculated. In larvae with *X. nematophila* OMPs, plasmatocytes and spherulocytes percentages were more than the negative control values and their trends in during time were irregular. In *X. nematophila* OMPs treatments there were no oenocytoids in the haemolymph of *S. exigua*. In larvae with *P. luminescens* OMPs treatments plasmatocyte density at 8-12 hpi was more than the negative control group. The trends of plasmatocytes were also irregular. Also, spherulocytes percentage from 2-16 hpi was more than in the control larvae with irregular fluctuation. The average of oenocytoidse was less than 1 (shown in the supplementary data).

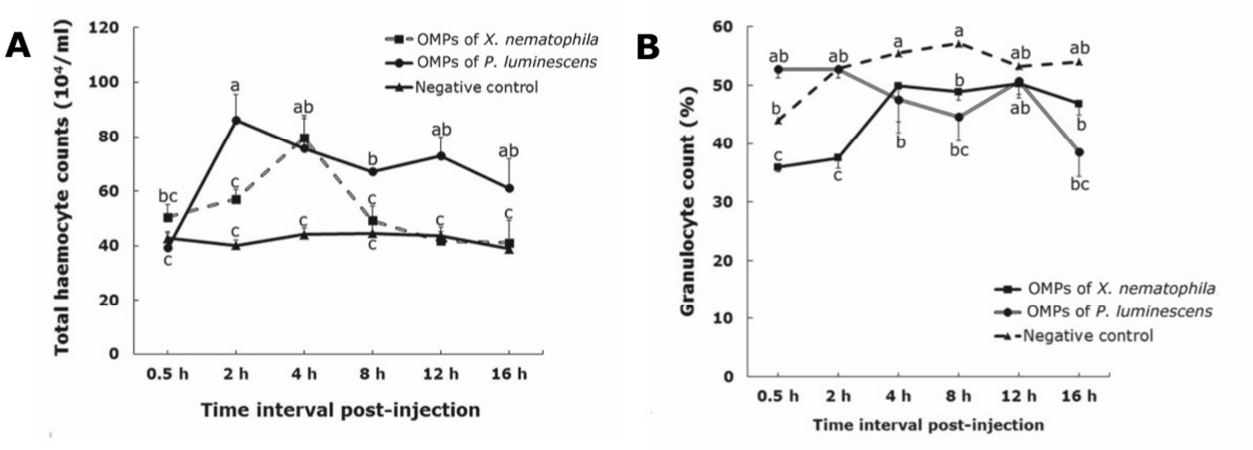

Figure 2. Changes pattern of cellular defense elements in fifth instar larvae of *Spodoptera exigua* after injection with outer membrane proteins of either *Xenorhabdus nematophila* or *Photorhabdus luminescens*, A) total haemocyte count, B) granulocyte percentage. Each measurement consists of eight replications. The vertical bars represent the standard error of the means. Different letters above the error bars indicate a significant difference of interactive effect means between outer membrane proteins types × interval times at α = 0.05 (Slicing test).

3.4. General protease activity

The interactive effect between OMP types and intervals time was significant ($F_{10,54} = 2.63$, $P < 0.05$) both OMP types ($F_{2,54} = 68.06$, $F < 0.05$) and time interval ($F_{5,54} = 4.72$, $F < 0.05$) on protease activity (Fig. 3A). Larvae with OMPs of *P. luminescens* exhibited an increase protease activity from 0.5 hpi reaching a maximizing level at 8 hpi and then decreased. This activity was higher than in the negative control insects during this time interval (0.5 h: $F_{1,6} = 28.12$, $P < 0.05$; 2 h: $F_{1,6} = 23.25$, $P < 0.05$; 4 h: $F_{1,6} = 77.99$, $P < 0.05$; 8 h: $F_{1,6} = 203.06$, $P < 0.05$; 12 h: $F_{1,6} = 9.71$, $P < 0.05$; 16 h: $F_{1,6} = 0.26$, $P > 0.05$). In larvae with *X. nematophila* OMPs protease activity was statistically compare with control larvae except for the absence of an increase at 4 hpi and a decline by 16 hpi (4 h: $F_{1,6} = 24.44$, $P < 0.05$; 16 h: $F_{1,6} = 41.86$, $P < 0.05$) (Figure 3A). Although protease activity was significantly different between both OMPs types by 2-16 hpi (0.5 h: $F_{1,6} = 4.02$, $P > 0.05$; 2 h: $F_{1,6} = 26.51$, $P < 0.05$; 4 h: $F_{1,6}$

= 2453.23, $P < 0.05$; 8 h: $F_{1,6} = 46.58$, $P < 0.05$; 12 h: $F_{1,6} = 31.02$, $P < 0.05$; 16 h: $F_{1,6} = 8.61$, $P < 0.05$). In larvae treated with both OMP types were observed the same pattern increasing from 0.5 to a peak at 8 hpi and thereafter declining albeit it at different rates ($P < 0.05$).

3.5. Phospholipase A_2 assay

Data show that the interaction between OMPs and time intervals exerted a significant effect on PLA_2 activity (Figure 3B). The PLA_2 volume in larvae with OMPs of *P. luminescens* although constant from 0.5 to 2 hpi, thereafter decreased and by 8 to 16 hpi the enzyme activity was less than the negative control. In larvae with *P. luminescens* OMPs exhibited statistically altered PLA_2 activity compared with control larvae except at 12 hpi at which time activities were similar a(0.5 h: $F_{1,2} = 32.23$, $P < 0.05$; 2 h: $F_{1,2} = 164.95$, $P < 0.05$; 4 h: $F_{1,2} = 287.99$, $P < 0.05$; 8 h: $F_{1,2} = 763.60$, $P < 0.05$; 12 h: $F_{1,2} = 862.95$, $P < 0.05$; 16 h: $F_{1,2} = 758.43$, $P < 0.05$). In larvae with *X. nematophila* OMPs, the PLA_2 level increased to a maximum level at 8 hpi and declined by 16 hpi to values less than the negative control levels (0.5 h: $F_{1,2} = 494.29$, $P < 0.05$; 2 h: $F_{1,2} = 1977.05$, $P < 0.05$; 4 h: $F_{1,2} = 3580.53$, $P > 0.05$; 8 h: $F_{1,2} = 174.28$, $P < 0.05$; 12 h: $F_{1,2} = 369.49$, $P < 0.05$; 16 h: $F_{1,2} = 2763.84$, $P < 0.05$) (Fig. 3B). There were a significant differences between the effect of both OMPs types on PLA_2 activity (0.5 h: $F_{1,2} = 136$, $P < 0.05$; 2 h: $F_{1,2} = 54.13$, $P < 0.05$; 4 h: $F_{1,2} = 7.16$, $P > 0.05$; 8 h: $F_{1,2} = 325.54$, $P < 0.05$; 12 h: $F_{1,2} = 312.46$, $P < 0.05$; 16 h: $F_{1,2} = 2.12$, $P > 0.05$); *P. luminescens* OMPs effect from 0.5 to 4 hpi being greater than *X. nematophila* OMPs and the latter being greater than the former from 8-12 hpi. Control values were constant throughout the test times.

3.6. Phenoloxidase assay

There was no evidence of significant interactive effect between OMP types and times on PO activity ($F_{10,54}$ = 1.43, P > 0.05) (Figure 3C). Control larvae exhibited a marginal increase in PO activity by 4 hpi, followed by a plateau. The control PO values were significantly less than either OMP types at all sample times. There was no significant difference in PO activity in larvae with *P. luminescens* OMPs versus control from 0.5 to 16 hpi (0.5 h: $F_{1,6}$ = 26.53, P < 0.05; 2 h: $F_{1,2}$ = 28.33, P < 0.05; 4 h: $F_{1,2}$ = 112.64, P < 0.05; 8 h: $F_{1,2}$ = 55.42, P < 0.05; 12 h: $F_{1,2}$ = 32.14, P < 0.05; 16 h: $F_{1,2}$ = 31.45, P < 0.05). However PO activity in larvae with *X. nematophila* OMPs increased gradually reaching a maximum value by 12 hpi and then decreased (0.5 h: $F_{1,6}$ = 29.66, P < 0.05; 2 h: $F_{1,2}$ = 31.30, P < 0.05; 4 h: $F_{1,2}$ = 983, P < 0.05; 8 h: $F_{1,2}$ = 113.08, P < 0.05; 12 h: $F_{1,2}$= 32.05, P < 0.05; 16 h: $F_{1,2}$ = 7.20, P < 0.05) (Figure 3C). There was no significant difference between OMPs of *X. nematophila* and *P. luminescens* on PO activation (0.5 h: $F_{1,6}$ = 0.05, P > 0.05; 2 h: $F_{1,6}$ = 0.77, P > 0.05; 4 h: $F_{1,6}$ = 1.11, P > 0.05; 8 h: $F_{1,6}$ = 0.03, P > 0.05; 12 h: $F_{1,6}$ = 1.75, P > 0.05; 16 h: $F_{1,6}$ = 1.34, P > 0.05).

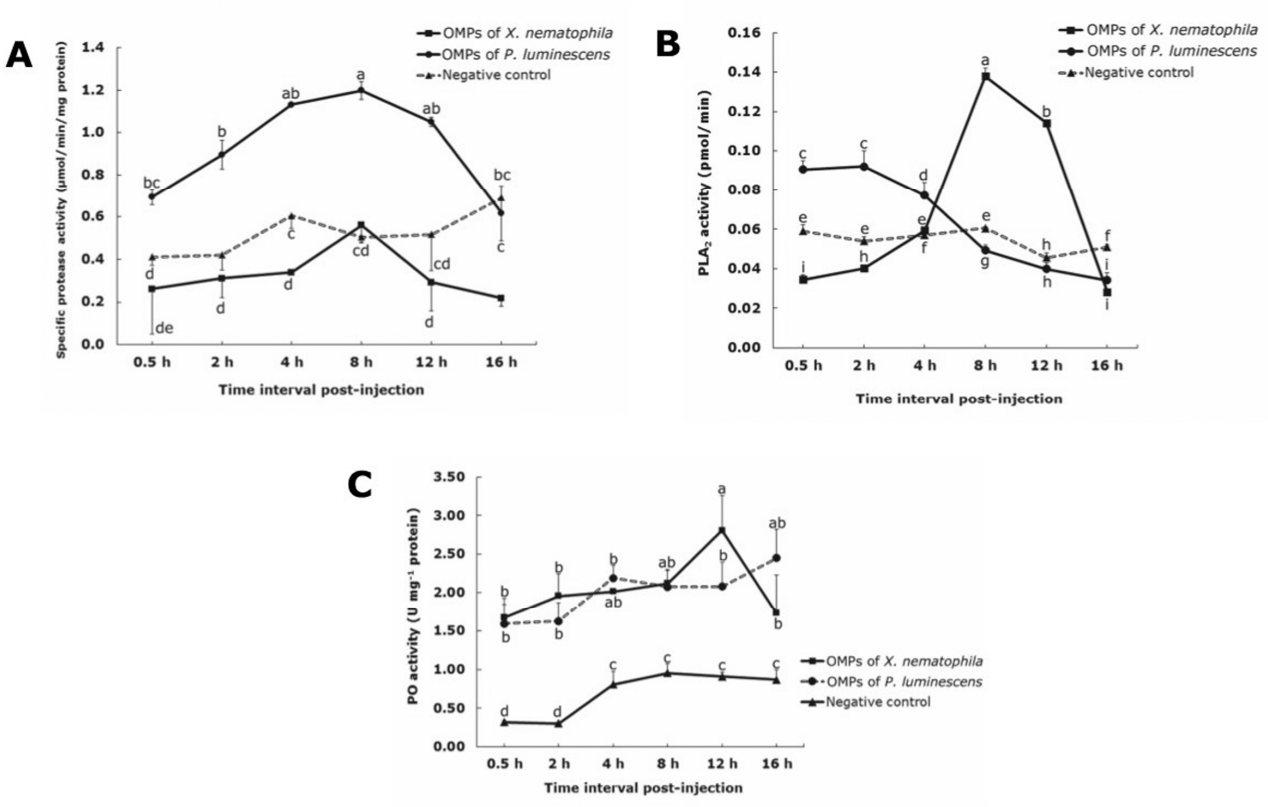

Figure 3. Changes pattern of humoral defense elements in fifth instar larvae of *Spodoptera exigua* after injection with outer membrane proteins of *Xenorhabdus nematophila* or outer membrane proteins of *Photorhabdus luminescens*, A) protease, B) phospholipase A2, C) phenoloxidase. Each measurement consists of eight replications. The vertical bars represent the standard error of the means. Different letters above the error bars indicate a significant difference of interactive effect means between outer membrane proteins types × interval times at α = 0.05 (Slicing test).

3.7. Attacin gene expression

The effect of OMPs of *P. luminescens* and *X. nematophila* on fluctuation of the attacin expression in *S. exigua* larvae was significant ($F_{6,12} = 255609$, $P < 0.05$) (Figure 4A). In larvae with *P. luminescens* OMPs, attacin expression value gradually increased from 2 hpi to a maximum level at 4 hpi (4 h: $F_{1,2} = 9446$, $P < 0.05$) then decreased by 8 hpi (16 h: $F_{1,2} = 3.36$, $P > 0.05$). Attacin expression in larvae with *X. nematophila* OMPs was less than those with OMPs of *P. luminescens* and reached a

maximum level with 6.10 ± 0.09-fold greater than the normal sample (non-injected sample) by 2 hpi. Thus the attacin gene was upregulated by both OMP types but, the degree of upregulation varying with the OMP types. The gene expression in injected larvae with control buffer was 0.39-0.5 times higher than the normal sample. There was a significant difference between the effect of both types on attacin expression (2 h: $F_{1,2} = 477.80$, $P < 0.05$; 4 h: $F_{1,2} = 9308$, $P < 0.05$; 8 h: $F_{1,2} = 6009.70$, $P < 0.05$; 16 h: $F_{1,2} = 1403.56$, $P < 0.05$) (Figure 4A). Both OMPs types were able to decrease attacin expression.

3.8. Cecropin gene expression

The effect of OMP types on fluctuations of cecropin expression in *S. exigua* larvae during the time was significant ($F_{6,12} = 17.32$, $P < 0.05$) (Figure 4B). In larvae with *P. luminescens* OMPs, cecropin expression elevated gradually from 2 hpi to the highest level at 4 hpi (4 h: $F_{1,2} = 1921.02$, $P < 0.05$) and then decreased (8 h: $F_{1,2} = 0$, $P > 0.05$; 16 h: $F_{1,2} = 38.27$, $P < 0.05$). The expression pattern of the cecropin gene was irregular in larvae with *X. nematophila* OMPs (2 h: $F_{1,2} = 4.90$, $P > 0.05$; 4 h: $F_{1,2} = 2.38$, $P > 0.05$; 8 h: $F_{1,2} = 3.16$, $P > 0.5$; 16 h: $F_{1,2} = 88.40$, $P < 0.05$). Cecropin expression in larvae with OMPs *X. nematophila* was less than those of treated with *P. luminescens* OMPs at the maximum value it was 18.52 ± 1.81-fold greater than the normal sample by 2 hpi (2 h: $F_{1,2} = 4.79$, $P > 0.05$). The cecropin expression in injected larvae with both OMPs was positive. However, the ability of OMPs of *P. luminescens* in decrease of cecropin expression was more than *X. nematophila* OMPs from 8 to 16 hpi. The gene expression in larvae injected with control buffer was 0.03-0.05 times higher than the normal sample. There was a significant difference between OMPs of *X. nematophila* and *P. luminescens* on cecropin expression at 4 and 16 hpi (2 h: $F_{1,2} = 44.8$, $P < 0.05$; 4 h: $F_{1,2} = 122.07$, $P < 0.05$; 8 h: $F_{1,2} = 31.6$, $P < 0.05$; 16 h: $F_{1,2} = 91.96$, $P < 0.05$) (Figure 4B). Both OMPs types were able to decrease cecropin expression.

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3.9. Spodoptericin gene expression

There was a significant interactiveeffect between OMP types and time ($F_{6,12} = 225$, $P < 0.05$). Also, OMPs type ($F_{2,12} = 193.14$, $P < 0.05$) during different times ($F_{3,12} = 216.39$, $P < 0.05$) had significantly affected the expression of spodoptericin expression (Figure 4C). Spodoptericin expression in treated larvae with *P. luminescens* OMPs in compared with the negative control reached the maximum level by 4 hpi (4 h: $F_{1,2} = 873.98$, $P < 0.05$) and then decreased (8 h: $F_{1,2} = 10.45$, $P > 0.05$; 16 h: $F_{1,2} = 47.82$, $P < 0.05$). In treatment with *X. nematophila* OMPs, a gradual decrease of spodoptericin occurred from 2 to 16 hpi (16 h: $F_{1,2} = 3.15$, $P > 0.05$). The change of spodoptericin gene activity was positive revealing that gene is up-regulated (Figure 4C). Both OMPs types were able to decrease spodoptericin expression. The trend of induced spodoptericin in larvae with *X. nematophila* OMPs and *P. luminescens* OMPs differed.

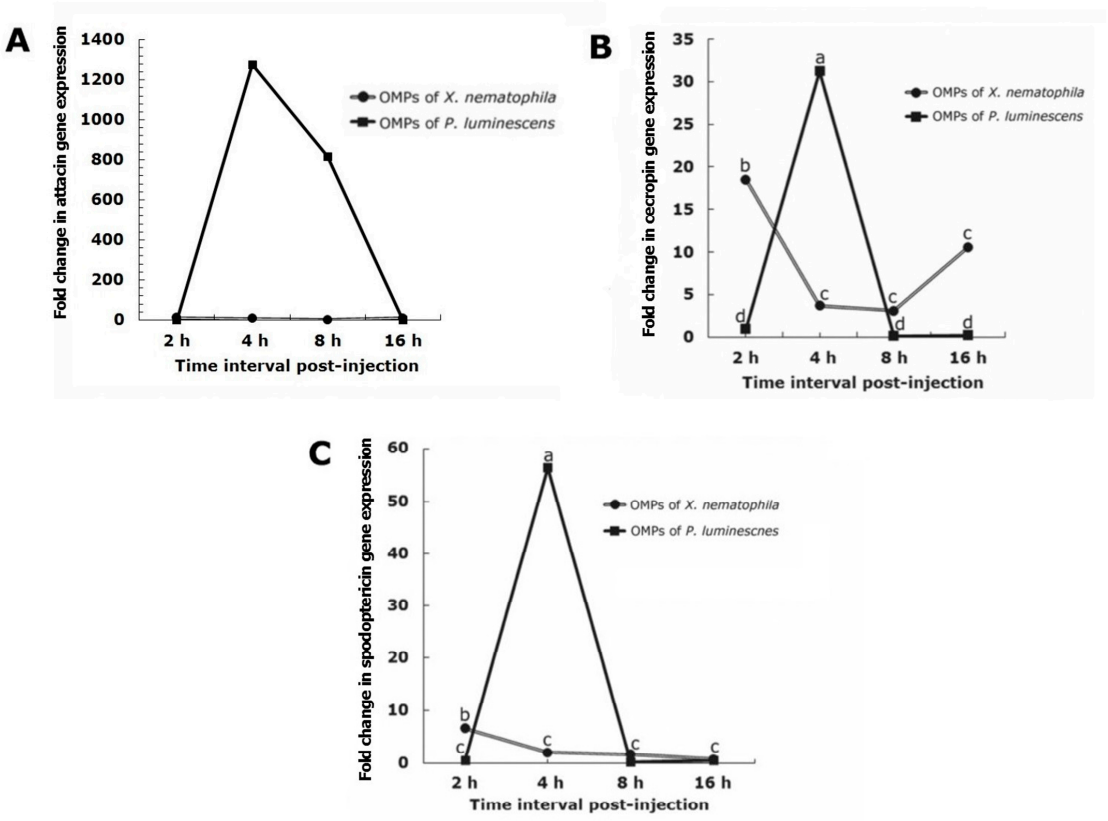

Figure 4. Changes pattern of genes expression in fifth instar larvae of *Spodoptera exigua* after injection with outer membrane proteins of *Xenorhabdus nematophila* or outer membrane proteins of *Photorhabdus luminescens*, A) attacin, B) cecropin, C) spodoptericin. Each measurement consists of eight replications. The vertical bars represent the standard deviations of the means. Different letters above the error bars indicate a significant difference of interactive effect means between outer membrane proteins types × interval times at $\alpha = 0.05$ (Slicing test).

4. Discussion

In the current study, the cellular and humoral aspects of the immune system in *S. exigua* larvae against purified OMPs of *X. nematophila* and *P. luminescens* were uniquely considered. The results imply that OMPs of the bacteria were able to modulate both the cellular and humoral defenses, the different OMP responses representing different types or amount of the modulating components. The study presents evidence that OMPs of the symbiotic bacteria contain components that likely contribute to their virulence. Which is similar to Rollauer *et al*. (7) the OMPs of mammalian pathogenic bacteria serve as virulence elements for evasion of the immune of the host Interesting and puzzling is that gene expression was influenced sooner than cellular and early stage humoral factors even though humoral cytokines activate traditional cellular responses or, by influencing cellular signaling pathways, elicit AMPs independently (25). The results indicate that the THC and granulocyte levels of *S. exigua* after injection of *X. nematophila* OMPs and *P. luminescens* OMPs decreased at 2 and 4-hour post infection, respectively. This could be attributed to OMPs eliciting apoptotic symptoms in the haemocytes because of the LPS releasing (26). The decline in the density of all haemocyte types in *Galleria mellonella* Linneaus larvae resulted from the lipid A moiety of *X. nematophila* and *P. luminescence* LPS action triggering haemocytes lysis (including oenocytoids) and inhibiting PO activation but not activity (27). Herein, OMP components activate PO directly or indirectly by lysis oenocytoids, releasing the enzyme, such release occurs on *S. exigua* (28). Additionally there was no correlation between PO activity and

oenocytoid density. In the treated larvae with OMPs of *P. luminescens*, PO activity increased at 0.5 to 16 hpi but in the treatments with *X. nematophila,* PO increased through 0.5-12 hpi and then decreased. We assume the decrease PO in larvae with *X. nematophila* OMPs was caused by OMPs. Collectively these results imply that there was no physiological amounts of LPS on the purified OMPs in the present study. Protease and PLA_2 were activated by both OMP types. In treated larvae with OMPs of *X. nematophila*, the protease activity at the beginning of injection was less than the negative control. This might this be a dual mechanism in which suppression and activation might linked with rearrangement of OMPs components over time.

Park & Kim (11) and Park et al. (10) confirmed that intact *X. nematophila* was able to decrease PLA_2 activity limiting cellular immunity. Herein OMP types were able to suppress defensive enzymes but fluctuation patterns of the PO, protease, and PLA_2 varied with OMP types. The differences could be related to variations in the types of OMP components and or their amounts and biological properties. Here it is confirmed that protein profiles of *X. nematophila* and *P. lumineses* are different. The lower ability of *P. luminescens* OMPs on haemocytes destruction, inhibition of PO activity and decrease of some humoral elements in initial times are in accordance with Forst and Nealson (8) who indicated the surface of *Photorhabdus* spp. may be different from of *Xenorhabdus* spp. The importance of cell surface properties in the life cycle and phase variation of *Xenorhabdus* cells has been linked to identified outer membrane proteins such as fimbria (pilin) and flagella (29). Although the properties of OMPs, flagella in phase I and phase II cells, fimbria (pilin) protein of surveyed *X. nematophila* are known (30). The cell surface properties of *Photorhabdus* cells has been more limited to OMPs (8; 31; 32).

In *S. exigua* larvae the AMPs attacin, cecropin, and spodoptericin genes are activated by the OMPs. Bacterial LPS activates numerous types of AMPs in Lepidoptera (33). While these effects may confer resistance to the bacteria by the host insect, Duperthuy et al. (34), established that outer

membrane protein U (OmpU) of *Vibrio splendidus*, the oyster pathogenic bacterium, contributes to its virulence by making the bacterium resistant to antimicrobial peptides. Vanaja et al. (35), reported that OMVs of extracellular Gram-negative bacteria can deliver LPS into the host cells; however, the mechanism of LPS translocation remains unclear.

Herein reported that OMPS of both *X. nematophila* and *P. luminescens* upregulated attacin and cecropin but expression of these AMPs by *X. nematophila* OMPs was less than *P. luminescens*. This phenomenon may be due to differences in OMP composition. Spodoptericin is expressed in lepidopteran insect with Gram-positive bacteria (36). Whereas, in the present work, spodoptericin expressed after injection of OMPs from both Gram-negative bacteria.

The pronounced fluctuations of attacin and cecropin that occurred in larvae with OMPs of *X. nematophila* were irregular. Whereas, AMPs expression in larvae with *P. luminescens* OMPs reached the maximum level by 4 hpi and then decreased. According to Castillo et al. (37), the expression level of cecropinA1/A2 in *Drosophila* after infection by *Photorhabdus* decreased at 30 hours. The decrease in AMPs expression could reflect the ability of *Photorhabdus* to degrade the host AMPs. Of the cytotoxic phospholipase C, protease, proelastase, and hemolysins reported in OMVs of *P. aeruginosa, Proteus mirabilis* and *Serratia marcescens* (6), lower case hemolysin, as a group of pore-forming proteins, is known to destroy the membrane of eukaryotic cells (38). Insect AMPs are synthesized after direct and indirect recognition of pathogens by the haemocytes (39) and fat bodies (40). However, destruction of haemocytes by OMPs may have led to a reduction in AMPs expression. *P. luminescens* OMPs decreased haemocytes density by 4 hpi following this, AMPs expression inhibited. In *X. nematophila* OMPs total heamocyte population was less than *P. luminescens* from 8 to 16 hpi and generally AMPs expression in larvae with *X. nematophila* OMPs were many less than *P. luminescnes*. In *X. nematophila* OMPs treatments maximum AMPs expression was 20-fold greater than the normal sample but the AMP expression in *P. luminescens* OMPs reach more than 1200-fold.

Herein attacin expression was more than cecropin and spodoptericin, the latter two AMPs possibly being digested by bacterial protease. Previously, Mak *et al*. (41) purified peptides with 4 kDa molecular weight from *G. mellonella* as Gm anionic peptide, Gm proline–rich peptide, defensin, a defensin-like and cecropin D-like and demonstrated which these AMPs were sensitive to degradation by elastase B. Andrejko & Mizerska-Dudka (42), indicated a specify type of elastase B produced by *P. aeruginosa* digests cecropin B in *Hyalophora cecropia* Linnaeus". Elastase is a protease from OMVs of insect pathogenic *P. aeruginosa* (43). The secreted alkaline metalloprotease (PrtA) produced by *Photorhabdus* sp. with homologies to *P. luminescens* and *P. temperata* inhibits the activity of *G. mellonella* cecropins A and B (44). *Photorhabdus* PrtS also cleaves insect antibacterial peptides (45). Also, the alkaline protease of *P. aeruginosa* may be responsible for degradation/inactivation of inducible AMPs in *G. mellonella* (42). Live *Xenorhabdus* inhibits expression of lysozyme and (46), cecropin in *S. exigua* (47) and purified protease II from *X. nematophila* reduced 97% of the cecropin A (48).

Generally, humoral and cellular immune defenses of insects are cross-linked. The PLA_2 has an important role in eicosanoid biosynthesis of insects, the eicosanoids affecting aggregation of haemocytes, haemocyte migration and release of prophenoloxidase from oenocytoids (49). Thus, the effect of PLA_2 activity leads to a change in cellular and humoral reactions. Here, the OMPs of *X. nematophila* and *P. luminescens* decreased PLA_2 activity and probably prevented eicosanoid biosynthesis a since AMPs expression in *S. exigua* by eicosanoid pathway is inhibited by intact *X. nematophila* (50).

Herein the data about the involvement of OMPs of *X. nematophila* and *P. luminescens* on *S. exigua* haemocytes and the expression pattern of main AMPs during are novel for insect pathology. We proposed the role for OMPs in the destruction of haemocytes, modulation of plasma enzymes (PLA_2 and PO) as the main defense source of infected insect. Here, in addition to the haemocyte density and

PO activity at interval times, the differential effects of OMPs from *X. nematophila* and *P. luminescens* on the number of granulocytes, protease, PLA_2 activities, attacin, cecropin, and spodoptericin expression were surveyed and indicated the difference in likely virulent factors between the bacterial species.

In summary, cumulative information suggests that secretion of insect toxins, outer membrane proteins, other extracellular products, and the release of LPS molecules from the bacterial envelope lead to the death of the host. Also, the current work increased our knowledge about the ability of OMPs in the suppression of cellular and humoral defense of insects.

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