Induction, Isolation and Biological function of Excreted/Secreted Products released by entomopathogenic nematode Heterorhabditis bacteriophora

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Trabajo Fin de Grado Biomedicina

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Abbreviations

- AMPs: antimicrobial peptides
- AUC: area under the curve
- BSA: bovine serum albumin
- CPS: counts per second
- EPNs: entomopathogenic nematodes
- ESPs: excreted/secreted products
- H1: frozen homogenate
- H2: fresh homogenate
- Hb-ilys-1: *Heterorhabditis bacteriophora* putative lysozyme 1
- HBSS: Hank´s balanced salt solution
- Hb-ugt-1: *Heterorhabditis bacteriophora* uridine diphosphate-glycosyltransferase 1
- HH: heat-inactivated homogenate
- HH2: concentrated heat-inactivated homogenate
- IJs: infective juveniles
- Imd: Immuno-deficiency
- LB: liquid lysogeny broth
- MAMP: microbe-associated molecular pattern
- Mcf: “makes caterpillars floppy” toxin
- OD: optical density
- PBS: phosphate buffered saline
- PHL: *Photorhabdus asymbiotica* lectin
- Pir: *Photorhabdus* insect related
- PLL2: *Photorhabdus luminescens* lectin 2
- PO: phenoloxidase
- proPO: prophenoloxidase
- PRPs: pattern recognition proteins
- PVC: *Photorhabdus* virulence cassettes
- ROS: reactive oxygen species
- Sc-Asp155: *Steinernema carpocapsae* aspartic protease 155
- Sc-ELA: *Steinernema carpocapsae* elastase
- Sc-Sp-1: *Steinernema carpocapsae* serine protease 1
- Sc-Sp-3: *Steinernema carpocapsae* serine protease 3

- SDS-PAGE: sodium dodecyl sulfate polyacrylamide gel electrophoresis
- Tcs: toxin complexes

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1. Abstract

Entomopathogenic nematodes (EPNs) are a type of parasitic nematode characterized by the development in insect hosts and symbiotic relationship with specific bacterial species. The EPN *Heterorhabditis bacteriophora* has a symbiotic association with bacterium *Photorhabdus luminesces*. During the infection of the host, the EPNs produce a variety of molecules known as Excreted/Secreted Products (ESPs) that have been described with immunomodulatory functions. Although EPNs only infect insects, humans and insects share analogous immune reactions of the innate immune system. Characterized ESPs from *H. bacteriophora* have the capacity to down-regulate the production of antimicrobial peptides (AMPs), induced by insect host hemolymph during infection. In addition, *P. luminesces* produces lectins that decrease the production of reactive oxygen species (ROS) in human blood phagocytes. In this study, we optimize the production process of *H. bacteriophora* ESPs in infective juveniles (IJs), by getting rid of contamination and using a variety of materials that induce their production. The different activating materials used come from a variety of compounds from insect host *Galleria mellonella*, which we name as homogenate. Furthermore, we isolated these ESPs and analyzed their immunomodulatory functions in insects and humans. We confirmed that the contamination found in the production of ESPs was due to Gram-positive and Gram-negative bacteria, and that this contamination could be eliminated with kanamycin (50 mg/ml) and streptomycin/penicillin (10,000 U/ml). Moreover, the use of these antibiotics showed an increase in the protein concentration of ESPs. We did not detect any increase in the protein concentration of ESPs, by the addition or removal of 0,01 % NaClO, nor with activation or secretion times selected in this study. An increase in the protein concentration of ESPs was observed when using as activating material concentrated heat-inactivated homogenate. We analysed the effect of ESPs over the antimicrobial activity of hemolymph from *G. mellonella,* by viability of bioluminescent *Escherichia coli*. We could not conclude if ESPs impede the antimicrobial activity of hemolymph or served as nutrients for the bacteria, due to lack of statistical significance. Lastly, we decided to measure the effect of ESPs over the production of ROS in human blood, concluding that collected ESPs do not significantly affect this type of immune response. This study benefits the ongoing research of bioactive ESPs over the areas of pharmacology, biocontrol and human medicine.

2. Introduction

Nematodes are a very large animal group, whose members can be found all over the world living in a variety of conditions. There are represented with insect parasites, human parasites, plant parasites and also with non-parasiting species like *Caenorhabditis elegans*. Entomopathogenic nematodes (EPNs) are a type of parasitic nematode characterized by the development in insect hosts and symbiotic relationship with specific bacterial species. EPNs are known to infect insects, and serving as mobile vectors for their insect-pathogenic bacteria cargo [1]. As mentioned, not all nematodes infect insects, there are for instance other species called filarial nematodes that can cause the infectious tropical disease filariasis (in humans known as lymphatic filariasis). This relationship between the nematode and the bacteria is mainly used in mutual benefit, due to the fact that the infection of insects is not only caused by the nematode but also by the bacteria. One of the stages of the life cycle of EPNs is the infective juvenile (IJ), which is the only stage living independent of the host in the soil [2]. This non-feeding IJ stage has the ability to infect the insects [3]. Once the EPNs are inside the host they release their symbiotic bacteria and start to develop, feeding on nutrient-rich hemolymph and insect tissues (**Figure 1**). Inside they are able to reproduce, feed themselves and lay eggs that will develop through the four main larval stages to another generation of adults. This cycle continues until nutrient sources in a host are depleted and then adult nematodes produce IJs as specialized larval stage, which abandon the insect cadaver in search of a new host to invade [4].

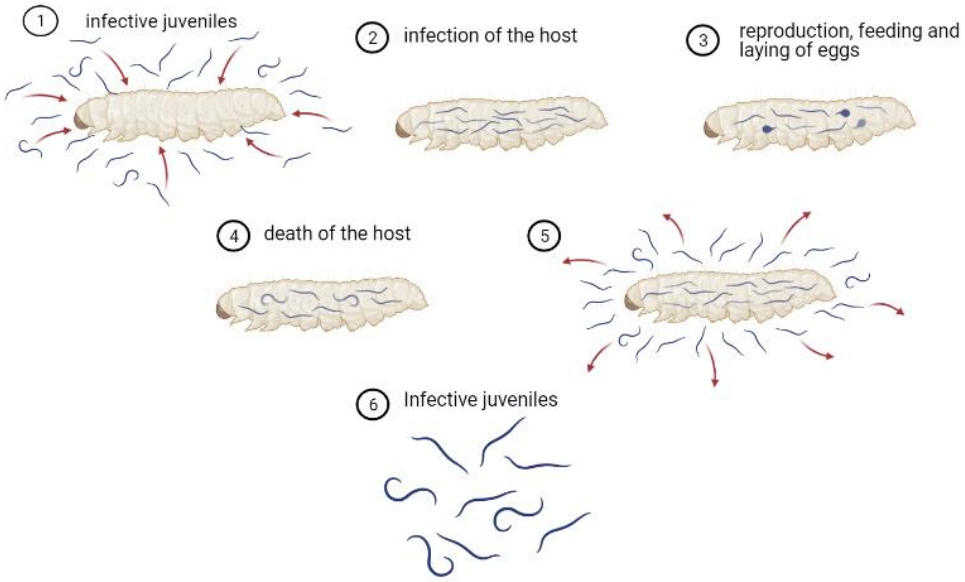

Figure 1. Life cycle of entomopathogenic nematodes. EPNs look for a potential insect host. They penetrate the host in the stage called infective juveniles (1) and once they are inside an insect, they start to immunosuppress the host by releasing excreted/secreted products and their symbiont bacteria to overcome its immune system and cause its death (2). The EPN develops

and feeds inside the insect (3). Once the hosts cadaver (4) is depleted of nutrients, they come out of the insect in another developmental stage called infective juveniles (IJ), which have the capacity to live freely in soil (5,6). Created in BioRender (www.biorender.com).

We can find two families of EPNs, which are Heterorhabditidae and Steinernematidae, from these two groups there are two species which are frequently used in biocontrol and experimental studies, *Heterorhabditis bacteriophora* and *Steinernema carpocapsae* [2]. *H. bacteriophora* belongs to the rhabditid nematodes in the eurhabditid clade [3]. This nematode is characterized by its short generation time, high fecundity, small size, transparency and its simple genome; due to this, it is an easy and effective model to work with in the study of parasitism, symbiosis/mutualism, pathogenicity, vector-born disease and its application for biological control of insects as biopesticide. The symbiotic bacterium of *H. bacteriophora* is *Photorhabdus luminescens,* which lives in close contact with the nematode. Due to this symbiosis, IJs can transmit *P. luminescens* selectively to their host [3]. This bacterium is a Gram negative entomopathogenic enterobacterium with bioluminescent activity which belongs to the Morganellaceae family, the wide range of toxins and secondary metabolites produced by this bacterium allows to kill insect larvae in a couple of days [5-6]. *P. luminescens* has a dualistic life cycle in one of the phases it can symbiotically interact with the nematode and the second one is a pathogenic phase toward a wide range of insects [5] such as *Drosophila melanogaster* and *Galleria mellonella* [4]. It has been also identified that some species of *Photorhabdus* such as *P. asymbiotica* and *P. australis* have the capacity to infect humans [7].

G. mellonella or also known as the greater wax moth or honeycomb moth is one of the most used biological models for the study of EPNs infection. It is also used as a model to study microbial infections [8] and other biomedical studies such as virulence, biologically active substances, fungicides, host resistance or efficacy of antibiotics [9]. *G. mellonella* is an insect from the order Lepidoptera and family Pyralidae. There are many reasons why to use this insect in the study of *Heterorhabditis* and its symbiotic bacteria but it is important to highlight that their maintenance and production is easy and cheap, without the need of specific laboratory equipment or a high biosafety level [8].

The infection of EPNs to the insect causes an immune attack over the nematode, the innate immunity plays a role in the battle against the parasitic infection. The immune system of insects is represented by innate immunity consisting of humoral (e.g. synthesis of antimicrobial peptides) and cellular reactions response mediated by immune cells called hemocytes (e.g. phagocytosis and encapsulation). The most common reaction in the fight against nematodes is encapsulation, coagulation, melanization and synthesis of antimicrobial peptides (AMPs) [4].

Melanization is a specific immunological process carried out by insects, related to killing any microorganism that may be causing an infection. To carry out this process there must be a recognition of foreign microbial structures or an interaction between other pathogen-associated patterns and a receptor, which results in melanin production. These microbial structures are recognized by pattern recognition proteins (PRPs) of the host. PRPs bind to microbe-associated molecular pattern (MAMP) molecules, synthetized by the pathogen [4-10]. Once the interaction has happened, the phenoloxidase (PO) cascade or other immune response starts. The enzyme PO starts the process in an inactivated stage, which is called prophenoloxidase (proPO); it becomes active once the serine protease cascade is activated. ProPO is present in hemolymph and because of that is where the activation occurs. The final objective of PO cascade is to produce toxic products that will help in the reaction against the parasite. This reaction is related with hemolymph clotting (preventing from more infections or healing a wound) and accompanies encapsulation, that appears as a dark capsule that surrounds the pathogen that is causing the infection in the insect [10]. Nematodes such as EPNs can interfere with the PO cascade in order to suppress or exploit its activity and cause and infection in insect hosts [4].

One of the humoral reactions on insects is the massive production of AMPs to eliminate invading pathogens. Insect AMPs are small cationic molecules found in hemolymph that increase their concentration in response to infection [4]. The recognition of microorganism occurs in the initial steps of Toll and Immuno-deficiency (Imd) pathways, recognizing MAMPs by PRPs. The activation of these pathways leads to gene expression of AMPs. Specifically Toll and Imd pathways activate a dorsal-related immunity factor which is related to NF-kB proteins, resulting in the expression of AMPs such as *Drosomycin* (synthetized by *D. melanogaster*) or *Gallerimycin* (synthetized by *G. mellonella*). The type of AMPs that are synthetized depend on the type of microorganism (e.g. AMPs against Gram-positive bacteria are not the same that the ones against Gram-negative). Once synthetized AMPs are secreted to hemolymph they act as a defense reaction of the insect [11].

Cellular reactions of the immune system are characterized by processes such as phagocytosis. This process is essential for host defense, not only in insects also in all types of vertebrates. Phagocytosis in insects is performed by granular cells and plasmatocytes, which internalize pathogenic microorganism to end with the infection. The internalization of the microorganism starts with the binding of cell surface receptors to the target microorganism or to opsonic factors that are attached to the target. Opsonic factors in insects are for example complement-like opsonins. Phagocytosis in *G.*

mellonella is also performed by these cells which circulate in the hemolymph [12]. Once internalized, phagocytes destruct the microorganism by different compounds such reactive oxygen species (ROS) [13].

The insects that are infected by EPNs respond with their immune system. To overcome this attack and be successful the infection, EPNs and their symbiotic bacteria produce a mixture of products to overcome the immune system of the insect host [1, 2, 3, 6]. *P. luminesces* symbiotic bacterium of *H. bacteriophora* produces toxins and enzymes that are essential for a successful infection. These products contribute on decomposition of different structures [6] and they have immunomodulatory abilities [7]. Further, the products of *P. luminesces* prevent that the corpse of the animal gets infected by other type of bacteria due to its capacity of producing antibiotics and bacteriocins in the first phase of its life cycle [14]. The secretion processes for all of these products are different. The most characterized products of P. luminesces are toxins, but there have also been described proteases and lipases. From the four pathogenicity islands that *Photorhabdus* contains in its genome, three encode for toxins [15]. The classification for the toxins of *P. luminesces,* includes four groups: the toxin complexes (Tcs), *Photorhabdus* insect related proteins (Pir), the “make caterpillars floppy” toxins (Mcf) and the *Photorhabdus* virulence cassettes (PVC) [16]. The (Tcs) are also divided into four different complexes which are Tca, Tcb, Tcc and Tcd. Each of them has different functions and ways of action, Tcc has a toxin enzyme activity, Tcb acts as a linker between the components of Tca and Tcc [17]. Tca and Tcd also work as orally active toxins that are responsible for the insecticidal activity against *Manduca secta* and Tcb cytotoxically against *G. mellonella* [16, 17]. The most known Pir are PirA and PirB, they have an injectable activity and its similarities with δ-endotoxins of *Bacillus thuringiensis* have been studied in detail [18]. Regarding Mcf toxins we can find Mcf1 and Mcf2 that are encoded alongside with hemagglutinin-like proteins, promoting apoptosis in the midgut of the insect by possessing a BH3 domain that justifies its pro-apoptotic actions. PVC are putative toxins with similarities to toxins A and C from *Clostridium difficile*, these toxins can destroy insect hemocytes and also have similarities with proteins of the phage tail and proteins from other bacteria with a high pathogenically potential [18].

As previously commented, the genus *Photorhabdus* contains some species that have the capacity of infecting humans [7] not only insects. As with any other microorganism the immune system reacts to the infection of this bacterium with the innate and adaptative systems [7]. The innate immune response that appears in humans possess a great deal of similarities with the insect response. The immune response will start the production of AMPs (humoral response) and the activity of phagocytes [7]. In vertebrate

species some of these phagocytes have the capacity of producing ROS in response to microbial infections, due to oxidative burst [19]. The immune system will recognize the infection of *Photorhabdus* thanks to the interaction of PRPs and PAMPs. An example of PAMPs present in bacteria are O-methylated sugars, which interact with PRPs such as lectins [20]. Being this one example of the various process's lectins are involved in the immune response. Lectins are not only produced by the host, studies have shown that *Photorhabdus* produces lectins such as PLL2 and PHL [20]. These lectins have the capacity of immunosuppressing the host by inhibiting the production of ROS [20].

EPNs and their symbiotic bacteria produce a mixture of products that will assure the effectiveness of the infective process. However, there is evidence that some genera of EPNs such as *Heterorhabditis* and *Steinernema* have a role in affecting hosts by the production of immune modulators similar as their symbiotic bacteria [12, 21]. The products produced exclusively by EPNs are known as excreted/secreted products (ESPs) [21]. It can be said that the immunomodulatory properties that EPNs have, are due to ESPs. When describing the nature of these products it can be said that they have a varied nature, belonging to different protein families or even nucleic acids. The toxicity of EPNs has been proved by using axenic infective juveniles, i.e. the nematodes without their mutualistic bacteria; it has been demonstrated that axenic infective juveniles of *S. carpocapsae* are capable of killing insect hosts by using their own products [22]. This has been also demonstrated by evaluating the cell-free growth medium, in which the axenic infective juveniles were reared. This medium has a toxic effect towards insects due to the pathogenic effects of ESPs [22].

In these years many studies have characterized ESPs from different EPNs, for example from *S. carpocapsae*. The most abundant ESPs of *S. carpocapsae* that has been found are proteases. Usually the proteases have a fundamental role in parasitism. The proteases identified are from the serine, metallo and aspartic families of proteases [22]. Aspartic proteases in EPNs are used in different roles of nutrition such as the beginning of the degradation of hemoglobin in the host. They are also involved in tissue degradation in general terms [22, 23]. Pepsin, rennin, cathepsin D, endothiapepsin, and chymosin are some of proteases included in aspartic family. One of the aspartic proteases that have been isolated from *S. carpocapsae* is the pepsin-like aspartic protease Sc-Asp155 which is expressed during the parasitic process of the nematode [22]. Serine proteases are the proteases that have been most successfully identified by its proteolytic activity [24], in this family we can find chymotrypsin proteases that inhibit prophenoloxidase activity and prevents encapsulation in *G. mellonella* [25]. Other serine proteases of *S. carpocapsae* is Sc-Sp-1, which destroys mainly basal lamina and peritrophic lamina, it

hydrolyzes fibronectin, laminin, even collagen IV and products of high percentage in the basal lamina [24]. Another member of the serine protease family is the elastase-like serine protease which is an endopeptidase that degrades proteins such as elastin [26]. This is an example of these type of proteases that have been identified as Sc-ELA protease. But proteases are not the only compounds that these EPNs produce, there have been also identified apoptosis inducing factors that work actively in the process of parasitic nematodes infecting hosts, cooperating with serine proteases such as Sc-Sp-3 [23].

The other genus of EPNs that are interesting for the study of ESPs is *Heterorhabditis* sp., specifically *H. bacteriophora.* The characterization of ESPs from this nematode has been slower or less effective than the characterization done with *Steinernema carpocapsae*, this can be due to the fact that the *Steinernema* genus induces a higher rate of mortality in insects than *Heterorhabditis.* One of the many reasons why the study of their products should be as important as other studies is because this type of nematode belongs to Clade V of Nematoda, making them related to *Caenorhabditis elegans* and strongylids (group of parasitic nematodes that can infect vertebrates, including humans; causing strongyloidiasis) [27]. Being related to *C. elegans* means that all the genetic tools that have been applied in this nematode could be also applied in *Heterorhabditis.* The ESPs of *H. bacteriophora* that have been characterized, have shown different effects related to the immune system of insects such as inhibiting the Imd pathway causing the down regulation of Diptericin secretion, antimicrobial peptide against Gram negative bacteria; making it easier for *P. luminesces* to propagate, infect the host and cause a higher rate of mortality [28]. It has been also demonstrated that *H. bacteriophora* ESPs inhibit the PO cascade and Toll pathway, which makes it easier to overcome the immune system of the insect [21]. There is a variety of enzymes that belong to these ESPs, such as glycotransferases, lysozymes, serine carboxypeptidases and more. One of these products is the Hb-ugt-1 [29] which reduces the upregulation of AMPs by the inactivation of ecdysone, hormone produced in insects that regulates development and moulting. This inhibition of AMPs favors *P. luminesces* to resist more in the host. A lysozyme that has been identified is Hb-ilys-1, which is in charge of degradation of peptidoglycan in Gram-positive bacteria in the gut and hemolymph of the insect [27]. With this degradation, the gut flora of the insect gets damage and makes it difficult to activate the immune system. More proteases such as cathepsin and astacins have been identified in the role of degradation of host tissues and blood [27, 30].

Over the past years the identification of new ESPs of *H. bacteriophora* has been a growing area of study (**Figure 2**). Also, the immunological actions that these products

have in relation to toxicity, parasitism and different cellular responses. One of the main problems that we can observe with ESPs is their complexity. Only a small part of the *H. bacteriophora* ESPs spectrum has been characterized. This is due to a big number of technical difficulties regarding their study [31]. One of them is the limited protein concentration, therefore yields are lower than is needed for their further characterization. Due to these factors, an optimization of the production and extraction of the ESPs is needed, and it can be achieved by making the process as similar to the physiological production of ESPs as possible.

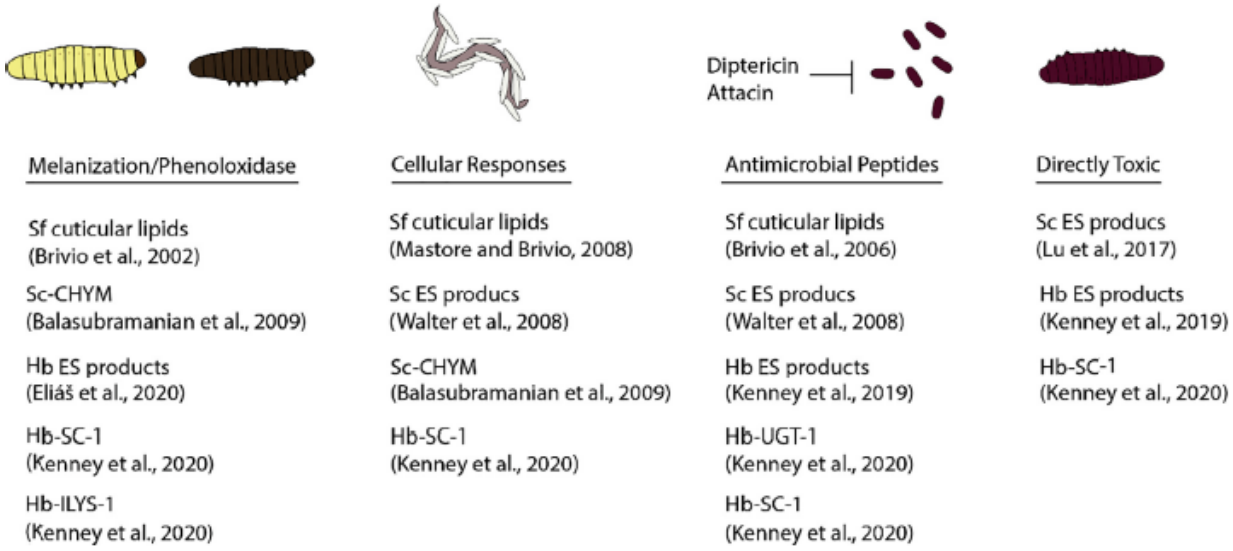

Figure 2. Different virulence products from entomopathogenic nematodes. *Steinernema feltiae* (Sf), *Steinernema carpocapsae* (Sc) and *Heterorhabditis bacteriophora* (Hb) attack in different ways their hosts to weaken their immunological system. They can be directly toxic, impair cellular responses, inhibit the phenoloxidase cascade or antimicrobial peptides. CHYM, chymotrypsin; ES, excreted-secreted; SC-1, serine carboxypeptidase; ILYS-1, invertebrate-type lysozyme; UGT-1, uridine diphosphate glycosyltransferase. Kenney *et al.* 2021 [27].

The aim of this study is to optimize the process of *in vitro* activation of *H. bacteriophora* IJs and isolation of their ESPs. Optimizing the collection process will result in obtaining bioactive compounds produced in the infection of *G. mellonella*. Thereby further characterization of ESPs can be done in order to be utilize in human medicine and study their pharmacological potential as bioactive molecules. Also testing the biological activity of the isolated ESPs by studying the roles these molecules have in different levels of the immune system has been done

3. Materials & Methods

3. 1. Insects and nematodes

G. mellonella or also known as the greater wax moth, was reared at 29°C in constant darkness and fed with an artificial diet according to Haydak [32].

The nematodes used were *Heterohabditis bacteriophora*, strain Az148, isolated in Azores, Portugal [33]. The insect larvae were placed in petri dishes that contained a layer of filter paper soaked with nematode suspension for 10 days at 25°C, where natural infection was developed [21]. Once infected, pigments produced by bacteria and EPN, turned the cadaver to characteristic red coloration. New IJs are released from cadavers approximately 10 to 14 days upon infection. White traps were used to collect IJs in a suspension [34] and several subsequent centrifugations (2 minutes at 500 g) to wash out dead IJs. Dead nematodes can be removed from suspension by pelleting their living associates and discarding supernatant. We stored living IJs at 11°C in the dark in a concentration of 25.000 IJs/ml of tap water. To calculate the nematodes concentration multi well slides and a stereomicroscope were used.

3. 2. Activation material

Frozen homogenate (H1) was prepared based according to D. Toubarro *et al* [25]. Briefly, 20 g of frozen wax moth larvae were grounded with a mortar and pestle until it became a thick paste. The paste was aliquoted into a 50 ml Falcon tube with 20 ml of Phosphate Buffered Saline (PBS, 137 mM NaCl, 2.7 mM KCl, 10Mm Na2HPO4, 1.8 mM KH2PO4, pH 7.4). Centrifugation at 5000 g for 15 minutes at 4°C was performed, to pellet the solid components of the homogenate. The supernatant was aliquoted into 15 ml Falcon tubes and stored at -80°C until further used.

Fresh homogenate (H2) was prepared in the same way as the frozen homogenate. Only the used *G. mellonella* were alive and the prepared homogenate was immediately used.

Heat-inactivated homogenate (HH) was prepared based according to previous studies [14, 35]. Specifically, 25 g of frozen *G. mellonella* larvae were grounded with a mortar and a pestle just as the standard homogenate. The paste was transferred to a glass beaker and mixed with 100 ml of PBS. The mixture was heated and boiled for 7 minutes and then cooled down at room temperature in a water bath. The homogenate was aliquoted in 50 ml Falcon tubes and centrifuged at 5000 g for 10 minutes at 25°C. The liquid supernatant including the oily layer was transferred in a new container. The process of centrifuging and adding PBS to the pellet was repeated until a volume of 100 ml of heat-inactivated homogenate was acquired. The aliquoted heat-inactivated homogenate was stored at -20°C.

The concentrated heat-inactivated homogenate (HH2) was prepared with the same protocol as the heat-inactivated homogenate, with the exception that it was not combined with PBS when added into the collection process.

PBS was used as a control for non-activated IJs, as this was the solution in which all the other materials were elaborate and it should not produce any activation.

3. 3. Collection process of ESPs from *H. bacteriophora*

This process was subjected to different changes depending on the activation material and the conditions measured. Even so, the main process utilized or optimized was the following. IJs stored at 11ºC for 14 days at the concentration of 25,000 IJs/ml were used. The suspension of IJs was collected into a Falcon tube having each tube a 40 ml suspension. The suspension was centrifuged at 500 g for 2 minutes. In the treatment with 0,01 % sodium hypochlorite (NaClO) this step followed. The supernatant was then removed and 40 ml of 0,01 % NaClO was added to surface-sterilize the IJs. The suspension was incubated for 10 minutes in an orbital shaker and three washings with PBS followed. The IJs were transferred to a Petri dish with 40 ml of PBS (in the case of concentrated heat-inactivated homogenate, 40 ml of undiluted activation material was used), 4 ml of activation material and antibiotics (Sigma-Aldrich, USA). The quantity of antibiotics added was 44 µl of ampicillin (50 mg/ml) and 44 µl of kanamycin (50 mg/ml) / 30 µl of kanamycin (50 mg/ml) and 30 µl of penicillin/streptomycin (10,000 U/ml). The suspension was incubated 2/6 hours at 25°C on shaker. After the specific time, the IJs were washed three times with 40 ml of PBS. The washed IJs were transferred to a Petri dish with 40 ml of PBS and antibiotics were added. The quantity added was 44 µl of ampicillin (50 mg/ml) + 44 µl of kanamycin (50 mg/ml) / 30 µl of kanamycin (50 mg/ml) + 30 µl of penicillin/streptomycin (10,000 U/ml), incubated on a shaker to secrete the ESPs. After 3 or 5 hours of incubation the suspension was filtered in acetate cellulose membrane 0,2 µm pore size (Sigma-Aldrich, USA). The final solution of ESPs was concentrated by centrifugation in Amicon centrifugal filter units (3kDa) (Merck, Ireland) using 5000 g to final volume of 200 µl. The ESPs concentration was quantified using a Quant-iT ™ Protein Assay Kit (Invitrogen, Molecular Probes Invitrogen detection technologies, USA) according to the instructions. The fluorescence was measured at 470/570 nm with spectrophotometer Sense (Hidex, Finland). Isolated ESPs were stored in -80°C for the further analyses. For an easier understanding, the collection process is schematized (**Figure 3**).

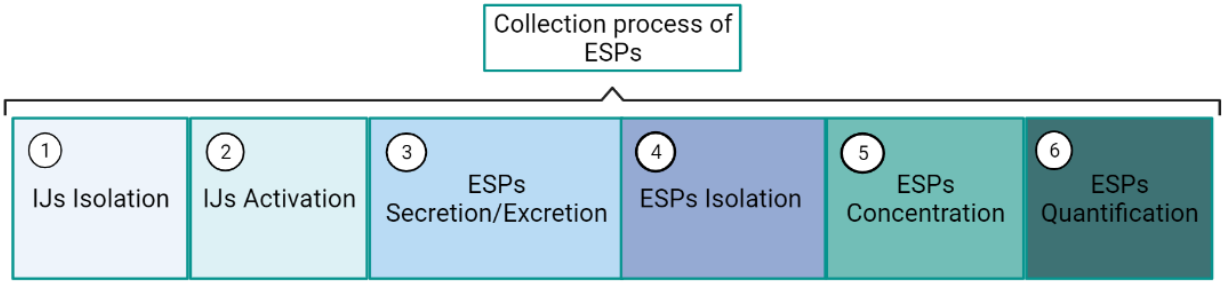

Figure 3. Collection process of ESPs from *H. bacteriophora*. (**1**) IJs were isolated at a concentration of 25,000 IJs/ml and if required treated with 0,01% NaClO. IJs were washed with PBS. (**2**) The activation consisted in making IJs produce ESPs by the addition of an activation material, along with antibiotics. The activation time selected was 2/6 hours. (**3**) In the secretion step, IJs were washed with PBS and antibiotics were added. The secretion time selected was 3/5 hours. (**4**) The isolation of ESPs was achieved by filtration in acetate cellulose membrane 0,2 µm pore size. (**5**) ESPs were concentrated to a final volume of 200 µl. (**6**) Quantification of ESPs was performed, obtaining the ESPs protein concentration (ug/ul), measured with fluorescence. Created in BioRender (www.biorender.com).

3. 4. Gram staining & MacConkey medium

The Gram staining was performed over samples taken from IJs suspension during the collection process to check for possible contaminants [36]. The samples were taken and transferred to a clean slide with drops of water to create a suspension. The suspension was air dried and heated. Crystal violet was poured and left in contact with the potential contaminants for 30 seconds and then rinse out with water. Gram´s iodine was added for 1 minute and washed with water. Washing with 95% alcohol was performed for about 20 seconds followed by a rinse with water. Safranin was poured for 1 minute and washed with water. The suspension was left to dry and observed under the microscope CX 31 (Olympus, Japan) with a magnification of 1000x. Images of the staining were taken with Levenhuk M500 BASE Digital Camera (5MPix) (Mikroshop, Czech Republic).

MacConkey medium was prepared according to manufacturer's instructions by mixing 5 grams of MacConkey Agar (Sigma-Aldrich, USA) with 100 ml of distillated water. Once the plates were prepared, aliquots from the contaminated samples were cultivated on this selective medium and incubated over night at 37°C. Pictures of the cultures were taken with Nikon Digital Camera D5300 (Nikon, Japan)

3. 5. Antibiotic sensitivity Assay

The grown colonies were taken from agar plates inoculated with samples collected during collection process. Contaminant colonies were subsequently transferred and cultivated in liquid lysogeny broth (LB) medium and incubated overnight shaking at 200 rpm and 37°C. The optical density (OD) of the bacteria was measured in a spectrophotometer (Tecan Sunrise, Switzerland) at a wave length of 600 nm. Dilutions were carried out to obtain a final OD of 1. In culture plates 25 ml of LB (cooled down

under 55ºC) were added, and 50 µl of bacterial suspension. Once the LB plates were solidified, three holes were made in the medium with a vacuum pump. Into the holes 30 µl of ampicillin (50 mg/ml), kanamycin (50 mg/ml) and streptomycin/penicillin (10,000 U/ml) were added. The culture plates were incubated over night at 37°C. The inhibition zone of each antibiotic in each plate was measured in mm.

3. 6. Antimicrobial activity Assay

The effect of ESPs over the antimicrobial activity of *G. mellonella* hemolymph, was measured by luminescence using bioluminescent *Escherichia coli* K12, which contains the plasmid luxABCDEamp for the expression of bacterial luciferase [37]. The assay was performed in a 96 well plate in which all the samples and controls were placed. For the dilution of 25 µl *E. coli* K12 (CFU 2,3x10^{6}) 3 ml of buffer solution were used, composed of potassium phosphate monobasic KH2PO4 (9,073 g/L) and disodium phosphate Na2HPO4 (23,9 g/L). Hemolymph of *G. mellonella* was collected from the prolegs and added into tubes containing phenylthiourea (PTU) to avoid melanisation. The reaction well had a final volume of 100 µl which was divided into 60 µl of *E. coli* K12 suspension and 40 µl of sample or buffer (control). The well with sample contained 5 µl of ESPs, 30 µl of buffer and 5 µl of hemolymph (final volume of 40 µl). For this assay a total of 3 controls were used: only buffer (40 µl); buffer (35 µl) with hemolymph (5 µl); and ESPs (5 µl) with buffer (35 µl). The luminescence was measured using Chameleon V luminometer (Hidex, Finland) for 110 minutes in counts per second (CPS). The integrals of the reaction were calculated, expressing the results as area under the curve (AUC). The data was normalized over the controls (buffer and buffer with hemolymph) for the presentation in the graphs.

3. 7. Reactive Oxygen Species Assay

The effect of ESPs over reactive oxygen species (ROS) in human blood was measured by collecting 2 µl of fresh human blood (type A was used for all the measurements), which was diluted into 173 µl of Hank´s buffered salt solution (HBSS; 0.137 M NaCl, 5.4 mM KCl, 0.44 mM KH2PO4, 0.25 mM Na2HPO4, 4.2 mM NaHCO3, 1.0 mM MgSO4, 1.3 mM CaCl2, 5.55 mM glucose, pH 7.4). To each well with this mix 10 µl of isolated ESPs were added with different concentrations (0.4 µg/µl, 0.2 µg/µl, 0.07 µg/µl) in Tyrode solution ((0.8% NaCl, 0.02% KCl, 0.02% CaCl2, 0.02% MgCl2, 0.005% NaH2PO4, 0.1% NaH2CO4, 0.1% C6H12O6). For the controls, bovine serum albumin (BSA; 0.4 mg/ml) and Tyrode solution were used instead of ESPs. The mix was incubated for 10 minutes, subsequently 25 µl of luminol (Sigma-Aldrich, USA; 10 mM) and 25 µl of Zymosan A (Sigma-Aldrich, USA; 2.5 mg/mL in HBSS) were added to activate ROS production. Luminescence was measured using luminometer Chameleon V luminometer (Hidex,

Finland) for 110 minutes hours at 37°C in counts per second (CPS) [20]. The integrals of the reaction were calculated, expressing the results as area under the curve (AUC). The data was normalized over the control (Tyrode) for the presentation in the graphs.

3. 8. Statistical Analysis

The statistical analyses were performed in software Prism (GraphPad Software version 8.0.1, USA). The data from the antibiotic sensitivity, ESPs protein concentration, antimicrobial activity and ROS production assay was evaluated using two-way ANOVA with Tukey´s multiple comparison test. In addition, the results of the antimicrobial activity and ROS production were evaluated by calculating the integral of the reaction (area under the curve, AUC). The normalization was included to get rid of individual variability of blood and hemolymph samples for a purpose of result figures. However, all data was statistically tested as mentioned above before the normalization. The statistical significance was considered significant with p values < 0.05 and error bars in each figure represent the standard deviation.

4. Results

4. 1. Optimization of ESPs collection process from *H. bacteriophora*

4. 1. 1. Contaminations in collection process

As the collection process should be free of any contamination, including bacteria; testing over the process was performed. Bacterial contamination was found in several steps of the collection process, identified with Gram staining and selective MacConkey medium. The results from the Gram staining showed that all of the samples collected from the process contained Gram-positive bacteria (**Figure 4A** – **C**) and only one of them presented Gram-positive and Gram-negative bacteria (**Figure 4D**). With the staining it was observed that the shape of the different bacteria was variable depending on the sample, Bacillus and coccus were found (**Figure 4A**, **B**). The same contamination was examined, with MacConkey medium. The results showed that in some of the agar plates, colonies had grown and in other plates not (**Figure 5**). Due to the characteristics of the medium it can be assured that the colonies that had grown, come from contamination of Gram-negative bacteria and the agar plates with no grown colonies were inoculated with contamination that came from Gram-positive bacteria. It can also be said that the Gram-negative colonies grown in the plates are non-lactose fermenting, due to the yellow-orange colour of the medium. With these results it can be confirmed that the contamination found in the activation process of IJs comes from not only Gram-positive but also Gram-negative bacteria.

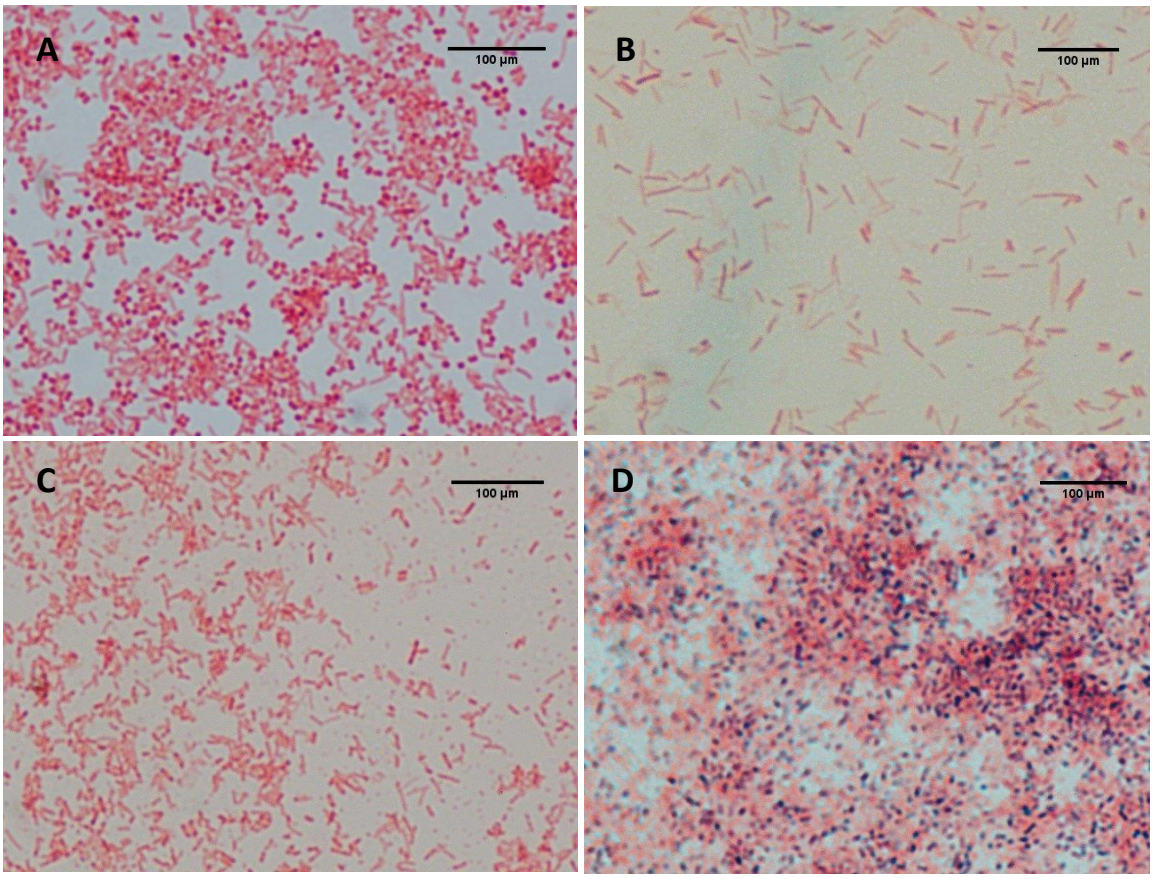

Figure 4. Gram staining of bacterial contaminants appearing during the IJs collection process. (**A - C**) Gram positive bacteria from the colonies grown in the agar plates inoculated with the contaminated aliquots of the IJs collection process with bacillus (**B**, **C**) and coccus (**A**) shape. (**D**) Gram-positive and Gram-negative bacteria from the colonies grown in the agar plates inoculated with the contaminated aliquots of the IJs collection process.

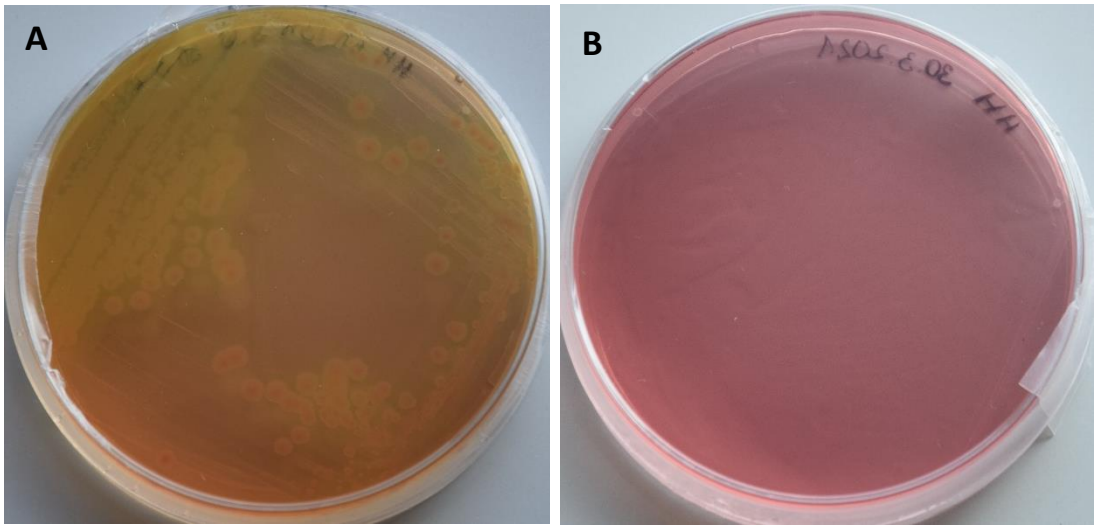

Figure 5. MacConkey medium culture plates with contaminated aliquots of the IJs collection process. (**A**) MacConkey medium culture plates with Gram-negative and non-lactose fermenting colonies. (**B**) MacConkey medium culture plate inoculated with Gram-positive.

4. 1. 2. Antibiotics used in the collection process

Based on the identification of bacterial contamination, three antibiotics with different concentration were tested with antibiotic sensitivity test. Three different bacterial colonies deriving from the agar culture plates with contaminated aliquots of the IJs activation process were chosen. These colonies were named as C1, C2 and C3. The inhibition zones of the selected antibiotics, ampicillin (50 mg/ml), kanamycin (50mg/ml) and streptomycin/penicillin (10,000 U/ml), were measured (**Figure 6**). The results showed that streptomycin/penicillin produced a significant effect over the bacterial contamination, compared to Ampicillin. Also, a higher effectivity of kanamycin 30.75 ± 3.18 and Streptomycin/Penicillin 34 ± 2.82 was observed in the three types of contamination. No contamination was observed after incorporating kanamycin and streptomycin/penicillin in the collection process (data not shown). With these results we can affirm that the antibiotics Kanamycin and the combination of Streptomycin/Penicillin produce a notable effect in eliminating the bacterial contamination of the IJs collection process.

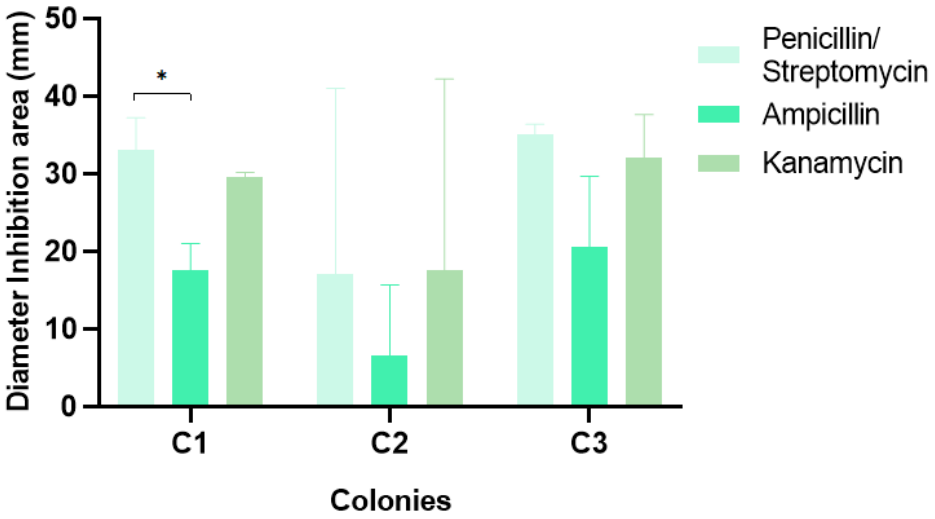

Figure 6. Inhibition area diameter of ampicillin (50 mg/ml), kanamycin (50 mg/ml) and streptomycin/penicillin (10,000 U/ml) from antibiotic sensitivity test towards three different bacterial colonies deriving from the agar culture plates with contaminated aliquots of the IJs activation process. C1, contamination present in all of the agar plates; C2, small white rounded colony; C3 big yellow colony. The brackets with asterisks show the significant differences $p < 0.05$ (Tukey´s multiple comparison test).

4. 2. Optimizing conditions of ESPs collection process from *H. bacteriophora*

4. 2. 1. Addition of kanamycin and streptomycin/penicillin to ESPs collection process

Protein concentration of ESPs obtained by the use of different combination of antibiotics in collection process was compared. With the results from the test (**Figure 6**), we initiated the collection process of ESPs with kanamycin and streptomycin/penicillin. As activation material for the process we used the different types of homogenate: H1, HH, HH2 and

PBS as a control. The measurements of protein concentration with the two combinations of antibiotics was carried out (**Figure 7**). The results showed that antibiotics kanamycin and streptomycin/penicillin produced a statistically significant increase of ESPs protein concentration in the combination with H1, HH and PBS.

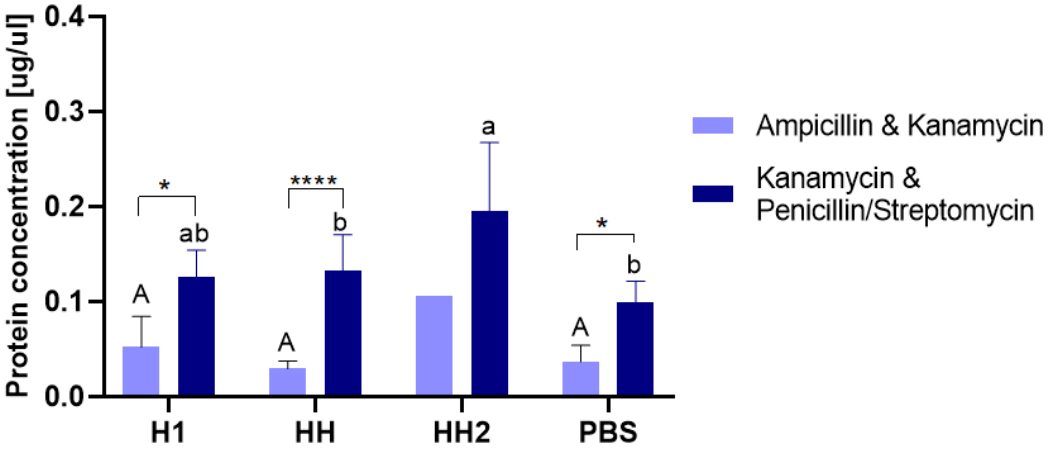

Figure 7. ESPs protein concentration of *H. bacteriophora* with the first combination of antibiotics [ampicillin (50 mg/ml) and kanamycin (50 mg/ml)], and with the second combination of antibiotics [kanamycin (50mg/ml) and streptomycin/penicillin (10,000 U/ml)]. All combinations of antibiotics were tested with IJs induced by a variety of materials: H1, frozen homogenate; HH, heat-inactivated homogenate; HH2, concentrated heat-inactivated homogenate and PBS, as control. The different letters above the bars and the brackets with asterisks show the significant differences $p < 0.05$ (Tukey´s test). HH2 treated with kanamycin and streptomycin/penicillin was excluded from the statistics since there was only one repetition of this treatment.

4. 2. 2. Effect of 0,01 % sodium hypochlorite (NaClO) in ESPs collection process

The chemical 0,01% NaClO was tested regarding how it affects presence of contaminants during collection process and resulting protein concentration of ESPs. The measurements were performed with and without NaClO as two different treatments, testing it over five different activation material: H1, H2, HH, HH2 and PBS as a negative control. The results obtained by the measurements of the ESPs protein concentration, showed that the use of NaClO with the combination of HH as activation material in the collection process produces a significantly higher protein concentration of ESPs (**Figure 8**). In other activation materials (H1, H2, HH2, PBS) any other effect of NaClO on the collection process was not observed.

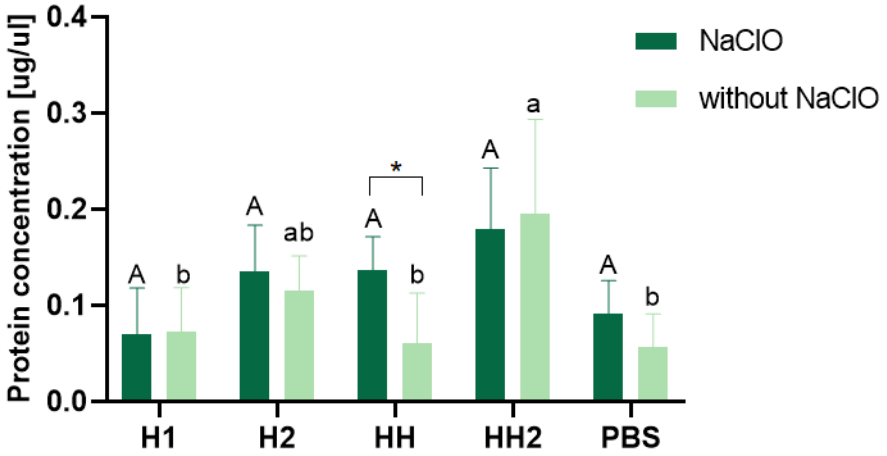

Figure 8. *H. bacteriophora* ESPs protein concentration comparing the effect of 0,01 % sodium hypochlorite (NaClO) over the ESPs collection process. The different groups used for the activation process were divided depending on the activation material used: H1, frozen homogenate; H2, fresh homogenate; HH, heat-inactivated homogenate; HH2, concentrated heat-inactivated homogenate and PBS, control. The different letters above the bars and the brackets with asterisks show the significant differences $p < 0.05$ (Tukey´s multiple comparisons test).

4. 2. 3. Variations in activation time of ESPs in the collection process

The effects over protein concentration of ESPs were compared with two variations of the activation time, which were 2 and 6 hours. These two time points were evaluated over five different activation material: H1, H2, HH, HH2 and PBS as control. The measurements showed that 2 hours or 6 hours incubation time of IJs with the different activation material had no significant effect in the ESPs protein concentration (**Figure 9**).

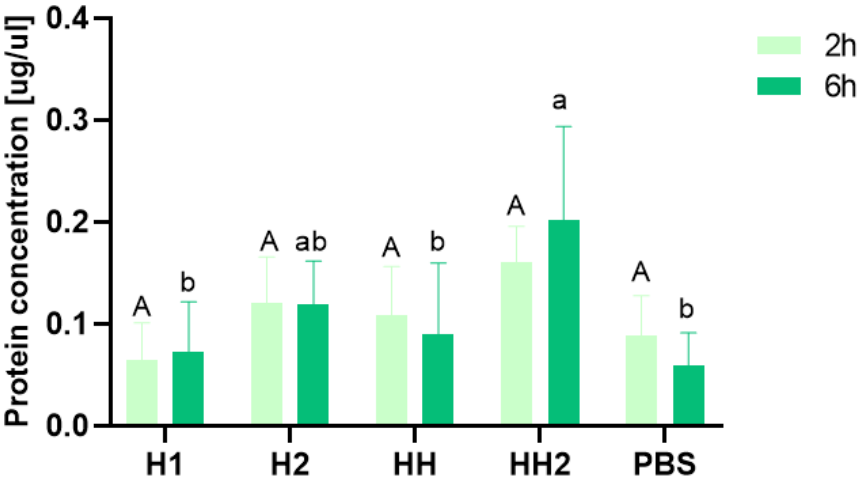

Figure 9. *H. bacteriophora* ESPs protein concentration after incubating 2 hours or 6 hours with different types of activation material: H1, frozen homogenate; H2, fresh homogenate; HH, heat-inactivated homogenate; HH2, concentrated heat-inactivated homogenate; PBS, control. The different letters above the bars show the significant differences $p < 0.05$ (Tukey´s multiple comparisons test).

4. 2. 4. Variations in secretion time of ESPs in the collection process

The protein concentration of ESPs was measured comparing two different time points of secretion time, which were 3 hours and 5 hours. These two time points were compared over five different activation materials: H1, H2, HH, HH2 and PBS as control. The measurements showed, that neither the 3 hour or 5 hour secretion time of ESPs produced any difference in the final protein concentration (**Figure 10**), producing no effect on any of the five activation materials.

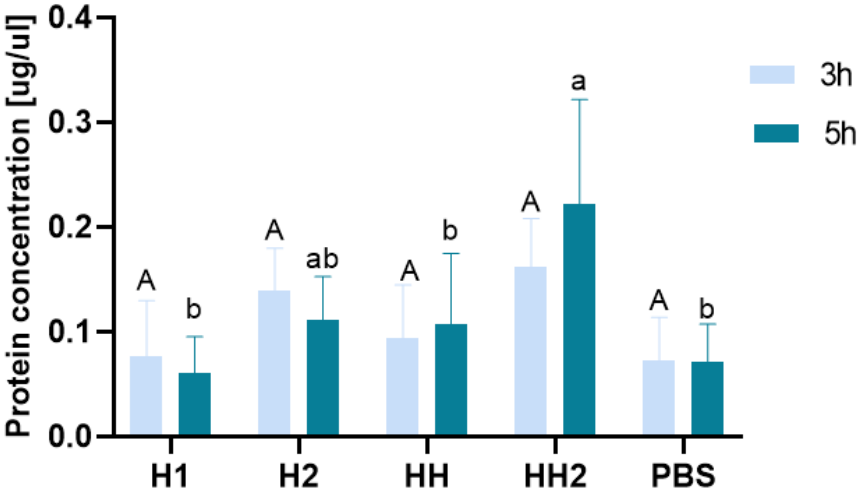

Figure 10. Protein concentration of ESPs with a secretion time of 3 hours or 5 hours in the isolation of ESPs process. Activation material used for different groups: H1, frozen homogenate; H2, fresh homogenate; HH, heat-inactivated homogenate; HH2, concentrated heat-inactivated homogenate; PBS, control. The different letters above the bars show the significant differences $p < 0.05$ (Tukey´s multiple comparisons test).

4. 2. 5. Different activation materials over ESPs collection process

The previous results showed that the effect of different activation materials was not the same over the different tested conditions. A difference was observed in activation material regarding the treatment with kanamycin and streptomycin/penicillin, NaClO, 6 hours activation time and 5 hours secretion time. The activation material HH2 with the combination of kanamycin and streptomycin/penicillin treatment, produced a significantly higher protein concentration than H1, HH and the control, PBS (**Figure 7**). The activation material HH2 in the treatment with NaClO produced a higher protein concentration of ESPs than H1, HH and PBS (**Figure 8**). Also, the activation time of 6 hours and secretion time of 5 hours had a higher protein concentration of ESPs when using the activation material HH2 (**Figure 9** – **10**). The results showed that the highest protein concentration of ESPs was achieved with activation material HH2.

4. 3. Biological Activity Analysis of ESPs over Immunity

4. 3. 1. Effect of ESPs on antimicrobial activity of *G. mellonella* hemolymph

The effect of five ESPs samples over the antimicrobial activity of hemolymph from *G. mellonella* was investigated; ESP 1 (protein concentration 0,234 µg/µl), ESP 2 (protein concentration 0,227 µg/µl), ESP 3 (protein concentration 0,195 µg/µl), ESP 4 (protein concentration 0,20 µg/µl) and ESP 5 (protein concentration 0,12 µg/µl). The assay using bioluminescence *E. coli* K12 was divided into treatment with hemolymph and without hemolymph. The results revealed that the mean of four of the ESPs samples treated with hemolymph had a higher inhibition of antimicrobial activity than the control hemolymph with bacterial buffer (H + B) (**Figure 11 A**), even though the difference measured was not statistically significant. The five samples without hemolymph showed an increasing trend of bacterial growth compared to bacteria with buffer (B) (**Figure 11 B**), however the differences were not statistically significant to conclude any result.

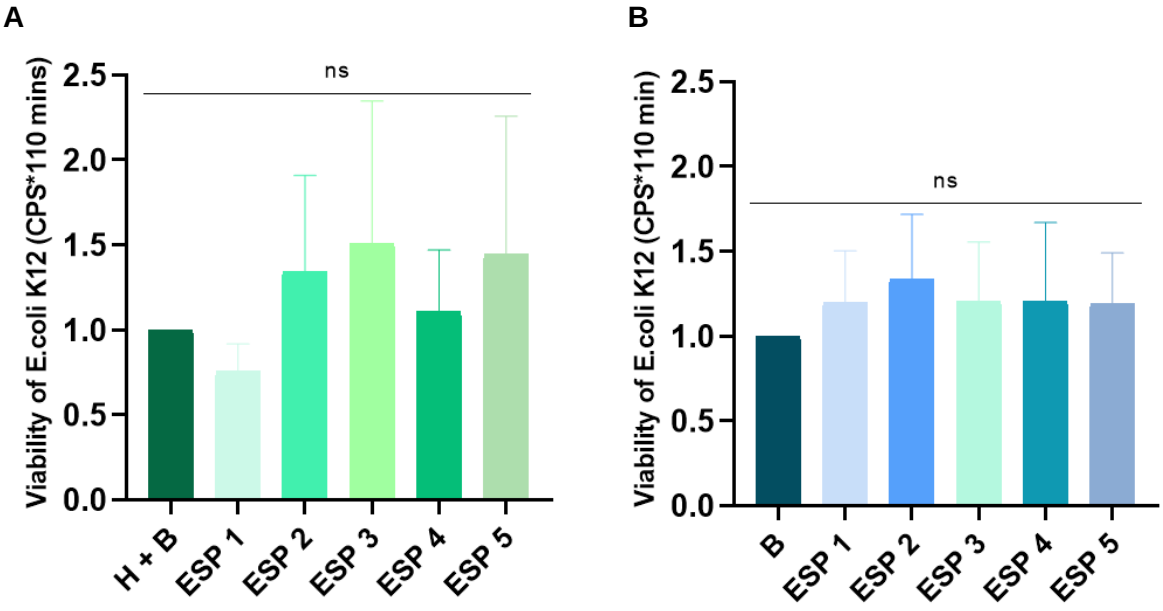

Figure 11. Effect of ESPs from *H. bacteriophora* on antimicrobial activity of *G. mellonella* hemolymph. (**A**) Measurements of bioluminescence measurement produced by *E. coli* K12, affected by ESPs samples treated with hemolymph (ESP 1, 2, 3, 4, 5); Control, buffer with hemolymph (H+B). (**B**) Measurements of bioluminescence measurement produced by *E. coli* K12, affected by ESPs samples without treatment with hemolymph (ESP 1, 2, 3, 4, 5); Control, buffer (B). Results are expressed as the integral of the reaction and normalized to the respective control. Not significant differences (ns) $p > 0.05$ between control and tested ESPs (Tukey´s test). N = 4.

4. 3. 2. Effect of ESPs on Reactive Oxygen Species (ROS) in human blood

To examine the recognition of ESPs by human immune system, the production of ROS was measured in presence of ESPs luminometrically. Three different samples of ESPs with different protein concentrations were used: ESP 6 (0.4 µg/µl), ESP 7 (0.2 µg/µl) and

ESP 8 (0.07 µg/µl). We used as negative control Tyrode and as protein control bovine serum albumin (BSA). The production of ROS was measured with two different conditions: constitutive production in spontaneously activated immune cells and in the presence of ROS production activator Zymosan. The results of the constitutive production of ROS revealed that the addition of ESPs caused an increase in the mean of ROS production compared to Tyrode and BSA (**Figure 12**). Although the trend in the graph is visible, the statistics showed that the difference in ROS production was not significant. The results of the treatment with zymosan showed that the addition of ESPs decreased the mean of production of ROS compared to Tyrode and BSA (**Figure 12**). Even so, the difference was minimal and we could not see statistical significance.

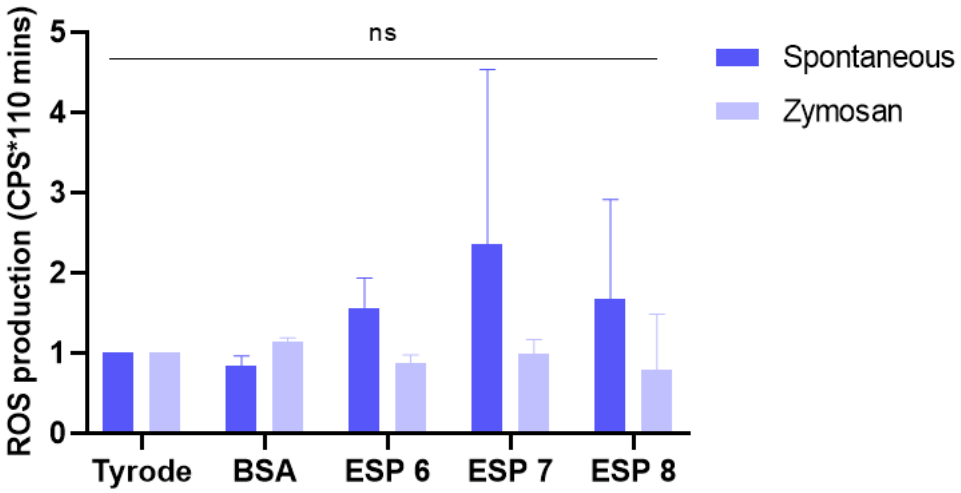

Figure 12. Effect of ESPs from *H. bacteriophora* on production of reactive oxygen species (ROS) in human blood. Measurements of ROS production by luminometry in the presence of three samples of ESPs: ESP 6 (0.4 µg/µl), ESP 7 (0.2 µg/µl) and ESP 8 (0.07 µg/µl). Controls: Tyrode and BSA. The assay measured effect on constitutive and zymosan-activated ROS production. The results were expressed as the integral of the reaction; and the data was normalized over to the control (Tyrode) of each treatment. Not significant differences (ns) $p > 0.05$ between control and tested ESPs (Tukey´s test). N = 2.

5. Discussion

This study focuses on isolation and characterization of ESPs released by entomopathogenic nematode *H. bacteriophora*. Since this nematode lies in symbiotic relationship with entomopathogenic bacterium *P. luminescens*, which is known for its extensive secondary metabolism and production of bioactive molecules, it is critical to perform ESPs collection under bacteria-free conditions. By using standard and selective media, a possible contamination of ESPs by Gram-positive and Gram-negative bacteria was confirmed as a risk factor (**Figure 4**, **5**). It is assumed that Gram-negative contaminants are related to bacterial symbionts of nematodes, however, the specific

species of contamination was not identified within the current study. Other contaminants, such as Gram-positive colonies, could be caused by commonly present bacteria, such as *Staphylococcus epidermidis* [38]. If a more detailed identification was to be made, we could perform some biochemical assays such as commercial biochemical kits that can provide us with enzymatic, biochemical and sugar tests for these bacteria. In our case, there was no need to continue with the identification of the bacteria due to the effectiveness of the antibiotics we tested. Although ampicillin, kanamycin and streptomycin/penicillin work against Gram-positive and Gram-negative bacteria, kanamycin and streptomycin/penicillin were the most effective ones (**Figure 6**). Kanamycin and streptomycin both inhibit the protein synthesis in bacteria by binding to 30S subunit of the ribosome [39], while ampicillin and penicillin inhibit the synthesis of cell wall by binding to penicillin-binding proteins [39]. A possible cause of why ampicillin was not as effective as the other antibiotics is due to antimicrobial resistance such as β-lactamases, affecting their mechanism of action [39].

Similarly, these antibiotics were used in the study as a condition to increase protein concentration of ESPs, which resulted effective (**Figure 7**). One of the aims of this research is obtaining only the ESPs of *H. bacteriophora*, but it is known that *P. luminesces* also produces some products [17, 18]. With the incorporation of antibiotics to the collection process of ESPs we not only eliminate possible contaminations that can interfere with the purity of the ESPs, we also make sure that the products that we are isolating are only from *H. bacteriophora*. As mentioned above, the antibiotics used act against Gram-positive and Gram-negative bacteria, therefore, *P. luminesces* as a Gram-negative bacterium should also be eliminated from the process. The increase in protein concentration of ESPs due to the use of the new antibiotics could be also justify with the fact that dead bacteria could leave small molecules or degradation material. One of the steps of isolating the ESPs is filtrating the suspension in an acetate cellulose membrane 0.2 µm pore size. The average size of bacteria is usually between 0.2 and 2 µm, but it has also been seen that some bacteria are able to pass through 0.1 µm filters [40]. Some degradation products could also pass through the filter and produce the increasement in protein concentration. To demonstrate if this was the reason why ESPs protein concentration had increased, mass spectrometry could be carried out. Previous to this study, mass spectrometry of ESPs from *H. bacteriophora* was performed, identifying protein domains that regulate Toll pathway and ubiquitination [21]. Bacteria identification and classification has already been achieved with mass spectrometry [41]. In addition, studies in parallel to this one in our laboratory used mass spectrometry analysis. This will be used also as a follow-up of this study and will help to identify specific ESP

components. In our study, mass spectrometry would identify the purity of the isolated ESPs, recognizing if products of bacteria are contained. It is also important to mention that while doing the comparison measurements of ESP protein concentration with both treatments of antibiotics, we did not use H2 as an activation material. This is due to the fact that when we started using H2 as activation material we already knew the effectivity of kanamycin and the streptomycin/penicillin on increasing the ESPs protein concentration.

Moreover, the effect of 0,01% NaClO in the collection process was tested due to two main reasons. Firstly, as a mechanism of nematode surface sterilization and secondly as a way to remove the old cuticle of the IJs [42, 43]. Until now the specific way how IJs of *H. bacteriophora* release the ESPs has not been described, removing the old cuticle (nematode exoskeleton made mainly of collagen, lipids, carbohydrates and glycoproteins) [4] is one of the markers of EPN activation and could allow IJs release a higher amount of ESPs. It has also been seen that different concentrations of NaClO affect the survival of IJs and damages them [43]. Due to these findings we decided to test how adding or removing NaClO from the activation process of IJs could possibly affect the protein concentration of ESPs. The results showed that adding or removing NaClO in the process had no significant effect on the protein concentration of ESPs (**Figure 8**). The only significant effect of NaClO over ESPs protein concentration was observed, when HH was used to activate IJs.

In order to examine all different conditions of the collection process, the variability in activation and secretion time was tested over the protein concentration of ESPs. EPNs need a period of time to get activated inside the host and start the production of ESPs. *H. bacteriophora* takes 1.5 hours to penetrate host *D. melanogaster* and approximately 6 hours for septicemia of the host due to release of *P. luminesces* [44]. Due to these results, we decided to measure the difference in protein concentration produced in 2 hours and 6 hours activation time, concluding that there was no significant difference in these interval time (**Figure 9**). Research in *S. carpocapsae* and *S. feltiae* shows that activation times of 6, 12, 18, 24 and 30 hours produce a notable difference in the production of ESPs, when IJs are exposed to an activating material such as host tissue (*G. mellonella* homogenate) [14, 35]. Furthermore, there is evidence that the 12 hours activation achieves a higher rate of activated IJs than 6 hours, 12 hours also leads to more developed nematodes and their ESPs are more toxic [14]. Although the time points we selected for the study tried to mimic the physiological process of infection and production of ESPs, longer activation times could produce a significant effect in protein concentration. Secretion time was another condition that was analyzed. Previous studies

in which ESPs of *S. carpocapsae* and *S. feltiae* were identified, used as secretion time 3 hours [14, 35] and 5 hours [28]. These secretion time points showed non-significant differences over ESPs protein concentration (**Figure 10**). A probable reason of why we cannot see difference in the time points is because the interval of time is only 2 hours, which could be not sufficient time to observe a difference.

To naturally initiate the production of ESPs, the nematode needs a stimulus that will simulate the infection of the host. Homogenate from *G. mellonella* as activation material was tested in different conditions to evaluate its effect over ESPs protein concentration. Frozen homogenate (H1) was the activation material used in the researches [21] that supported this study. Even though, the low protein concentration acquired lead us to hypothesize over new variations of this homogenate. H1 is stored at -80°C which means that to be used it has to be defreezed and it can be stored long periods of time, possibly affecting the biological properties of the material. H2 was the most appropriate option to test if the freshness of the activation material affected ESPs protein concentration. HH was used by other research groups that also studied ESPs of EPNs [14, 35]. This approach of heating the homogenate helps with the sterilization and trying to avoid contamination in the activation material, but also leads to denaturation of host proteins. In addition, concentration of the activating material could affect ESPs protein concentration, therefore HH2 was also examined. The results indicated that HH2 produced a significantly higher ESPs protein concentration (**Figures 7**-**10**). Even so, further studies of HH2 as activation material should be carried out. HH2 could produce a higher protein concentration due to the presence of *G. mellonella* small fragments in the homogenate, as this activation material has a more concentrated fraction of homogenate. This could be verified by performing an experiment that shows the different proteins in the ESPs solution, such as sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). With this type of electrophoresis, we could observe the proteins of the ESPs and compare it with the proteins present in the HH2. An interesting activation material could also be *G. mellonella* hemolymph as it circulates around all the body and is in contact with all the insect tissues [4]. Other studies related to *H. bacteriophora* and *S. carpocapsae* use hemolymph as activating material for their IJs [29, 45]. Although hemolymph favors a more physiological process possibly increasing ESPs protein concentration, we did not test its effect due to practical and time limitations.

ESPs are involved in a variety of immune reactions acting as immunomodulators due to the different biological activities they possess [21-31]. It has been confirmed that ESPs produce the downregulation of AMPs such as Diptericin, Attacin and Drosomycin, in hosts such as *D. melanogaster* [29, 30, 44]; causing a state of immunosupression. The

hemolymph of *G. mellonella* has also indicate to have antimicrobial properties because of the presence of AMPs [46]. Since there was evidence that ESPs have immunomodulatory functions, the effect of ESPs on antimicrobial activity in *G. mellonella* hemolymph was tested. The results suggested that the antimicrobial activity of hemolymph was being impede by the ESPs, however, this was not concluded due to the lack of statistical significance (**Figure 11 A**). Additionally, we tested the effect of ESPs directly on Gram-negative bacteria *E. coli*. Observing in the results an interesting trend, since we can see an increase in the luminescence during the assay (**Figure 11 B**). This could be explained by the fact that ESPs are a mix of proteins and different molecules that can serve as nutriment for bacteria, causing then a growth in the number of bacteria, which in turn produce stronger bioluminescence signal in our assay. Although there was not statistical significance that can insure this, the lack of significance can be explained with both of our hypothesis. The hemolymph of *G. mellonella* has an antimicrobial activity which would kill the bacteria, producing a decrease in luminescence. If ESPs nourish bacteria or by another mechanism impede the antimicrobial activity, luminescence increases as we see in the assay. These ups and downs in luminescence could be the cause of the lack of significance in the results. As future approximation another type of assay that would also test the activity of ESPs over antimicrobial activity could be done, to examine the trend observed in our study.

Bioactive compounds produced by nematode symbionts have been described, these have the capacity to impair ROS production by phagocytes in an early immune response in human blood [20, 47]. The immune-modulative activity of ESPs in insect host has been described [21-31], however studying the immunomodulatory capacities of ESPs in humans would mean a lot for future applications in medicine. Due to this, we decided to examine if ESPs affected fundamental cellular reactions, specifically phagocytosis by the measurement of ROS production. We observed that the mean of the constitutive ROS production when ESPs were added, was higher than the controls (**Figure 12**). This could be indicating the possible recognition of ESPs by the human immune system, triggering the immune response of phagocytes over the pathogenic molecules. This increasement in ROS could also contribute to damage of host tissue by the oxidative reactions. Even though, our results did not show statistical significance. We measured the production of ROS induced by ESPs in presence of zymosan, observing that the mean of the production levels was lower that the controls (**Figure 12**). A decrease in ROS production could indicate the ability of ESPs to interfere with the human immune response over ROS activator zymosan, however we did not observe any significant effect of ESPs. In addition, this could also indicate that ESPs interfere specifically with Toll-like receptors

type 2 (TLR-2), since zymosan, due to being a PAMP; is a ligand of TLR-2 [48]. Moreover, the possible interaction between ESPs and human immune system could help bacterial symbionts of EPNs overcome susceptibility to ROS produced by phagocytic cells. Although the trend of the results indicate that ESPs could have this immunomodulatory function over human immune system, we cannot conclude it due to lack of statistical significance.

The characterization of ESPs is a continuously growing area which contributes to the identification of specific molecules potentially with immunomodulatory functions. By achieving a higher protein concentration of ESPs, the study of their biological activity can be more effective and efficient. Further study of ESPs over different immune mechanisms will benefit areas such as pharmacology due to its already observed potential, possibly being used in human medicine or biocontrol.

6. Conclusions

In this study we optimized the collection process of ESPs from *H. bacteriophora* and analyzed different conditions of this process. The Gram-positive and Gram-negative bacterial contamination found in the process was eliminated by the addition of kanamycin and streptomycin/penicillin, achieving thereby a higher protein concentration of ESPs in the collection. Other conditions such as the addition of 0,01% NaClO and different time intervals of the collection process, did not affect positively or negatively to the protein concentration of ESPs. The variety of activating material, used to simulate the infection on *G. mellonella*; showed that concentrated heat-inactivated homogenate generates the highest protein concentration in ESPs. We also examined biological activities of ESPs regarding insect and human immunity. We conclude that with the results obtained we cannot observe any effect over the antimicrobial activity of the hemolymph from *G. mellonella* or over the production of ROS by immune cells in human blood, therefore their exact role in the infection process remains unknown. However, there is still a big number of ESPs undescribed, and with further research on their characterization and biological activity ESPs area is a promising area of studies.

7. Bibliography

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