ADRIANE TOLEDO BATISTA DA SILVA

Pleurotus spp. AND THEIR PROTEASES: A NEW APPROACH TO ALTERNATIVE HELMINTH CONTROL IN AGRICULTURE AND LIVESTOCK FARMING

LAVRAS – MG
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Tese apresentada à Universidade Federal de Lavras, como parte das exigências do programa de Pós-Graduação em Agroquímica, área de concentração em Bioquímica, para a obtenção do título de Doutora.

Orientador
Prof. DSc. Filippe Elias de Freitas Soares

Coorientador
Prof. DSc. Willian César Terra

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Aprovada em 10 de agosto de 2026.

DSc. Clara Resende de Souza Castro UFLA

DSc. Wilton Soares Cardoso IFES

DSc. Tatiana Cardoso e Bufalo UFLA

DSc. Rafael Neodini Remedio UFLA

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Prof. DSc. Filippe Elias de Freitas Soares

Coorientador
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Dedico este trabalho, primeiramente, a Deus, por sua graça,
cuidado e por me permitir chegar até aqui.
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“Consagre ao Senhor tudo o que você faz,
e os seus planos serão bem-sucedidos.”
Provérbios 16:3

RESUMO

O uso de cogumelos comestíveis e de seu extrato bruto proteolítico livre células, especialmente de espécies do gênero *Pleurotus,* como agentes de controle biológico e bioquímico, apresenta-se como uma alternativa sustentável para o manejo de parasitas na agropecuária. Nesse sentido, essa tese teve como objetivo geral otimizar a produção de enzimas por *Pleurotus* spp. e avaliar tanto a atividade antiparasitária dos fungos quanto de suas enzimas proteolíticas sobre ovos e juvenis de nematoides parasitas de plantas e larvas de parasitas gastrointestinais de animais. O primeiro artigo apresentou uma revisão das proteases produzidas por *Pleurotus* spp., abordando sua produção, propriedades bioquímicas e aplicações biotecnológicas. O segundo artigo investigou a atividade nematófaga de várias espécies do gênero *Pleurotus* e nematicida de suas proteases contra *Panagrellus* sp. Correlações significativas foram observadas entre ambos ensaios. O terceiro artigo avaliou a atividade nematófaga *in vitro* de *P. djamor* e nematicida de suas proteases sobre larvas infectantes de *Trichostrongylus* spp. e *Strongyloides papillosus*, evidenciando uma redução de 75% dos nematoides no ensaio nematófago e 57% no ensaio nematicida. No quarto artigo, foi realizada a otimização da produção de proteases por *P. djamor* usando fermentação em estado sólido com resíduos agrícolas, especificamente farelo de trigo. Os resultados indicaram que os níveis de umidade influenciaram significativamente a produção de enzimas. Ainda neste artigo, as proteases demonstraram atividade anti-helmíntica *in vitro* significativa contra ovos de *T. solium* e *Moniezia* sp., com reduções de 33,44% e 45,43%, respectivamente. O quinto artigo analisou o perfil proteolítico e a ação nematicida das proteases de *P. djamor* contra larvas de *Haemonchus* spp. e *Trichostrongylus* spp., resultando em uma redução significativa de 35% das larvas. A análise por zimograma revelou pelo menos duas proteases com pesos moleculares entre 75 e 100 kDa. O sexto artigo avaliou a atividade nematicida e ovicida das proteases de *P. djamor* sobre *Meloidogyne incognita in vitro* e em condições de estufa, demonstrando uma redução de 100% em juvenis e de 22% em ovos *in*

vitro. Tanto os extratos com proteases ativas quanto com proteases desnaturadas de *P. djamor* reduziram significativamente o número de ovos em 94% e 97%, respectivamente, além de diminuírem a formação de galhas em condições de estufa. Por fim, o sétimo artigo investiga o uso combinado de proteases do *P. djamor* e da papaína, visando o desenvolvimento de formulações mais eficazes para o controle de nematoides. Os resultados dos artigos evidenciam o potencial do fungo *Pleurotus* spp. e de suas proteases como alternativa sustentável para o manejo de parasitas importantes presentes agropecuária.

Palavras-chave: Enzimas; Cogumelo comestível; Controle bioquímico; Parasitas.

ABSTRACT

The use of edible mushrooms and their cell-free proteolytic crude extract, especially from species of the genus *Pleurotus*, as biological and biochemical control agents presents itself as a sustainable alternative for parasite management in agriculture and livestock. In this context, the general objective of this thesis was to optimize enzyme production by *Pleurotus* spp. and evaluate both the antiparasitic activity of the fungi and their proteolytic enzymes on eggs and juveniles of plant-parasitic nematodes and larvae of gastrointestinal parasites of animals. The first article presented a review of the proteases produced by *Pleurotus* spp., addressing their production, biochemical properties, and biotechnological applications. The second article investigated the nematophagous activity of several species of the genus *Pleurotus* and the nematicidal activity of their proteases against *Panagrellus* sp. Significant correlations were observed between both assays. The third article evaluated the *in vitro* nematophagous activity of *P. djamor* and the nematicidal activity of its proteases on infective larvae of *Trichostrongylus* spp. and *Strongyloides papillosus*, showing a 75% reduction of nematodes in the nematophagous assay and 57% in the nematicidal assay. In the fourth article, the optimization of protease production by *P. djamor* was carried out using solid-state fermentation with agricultural residues, specifically wheat bran. The results indicated that moisture levels significantly influenced enzyme production. Also in this article, the proteases demonstrated significant *in vitro* anthelmintic activity against eggs of *T. solium* and *Moniezia* sp., with reductions of 33.44% and 45.43%, respectively. The fifth article analyzed the proteolytic profile and the nematicidal action of *P. djamor* proteases against larvae of *Haemonchus* spp. and *Trichostrongylus* spp., resulting in a 35% reduction in larvae. Zymogram analysis revealed at least two proteases with molecular weights between 75 and 100 kDa. The sixth article evaluated the nematicidal and ovicidal activity of *P. djamor* proteases on *Meloidogyne incognita in vitro* and under greenhouse conditions, demonstrating a 100% reduction in juveniles and 22% in eggs *in vitro*. Both extracts containing active proteases and denatured proteases of *P. djamor* significantly reduced egg numbers by 94% and 97%, respectively, and decreased gall formation under greenhouse conditions. Finally, the seventh article investigates the combined use of *P. djamor* proteases and papain, aiming at the development of more effective formulations for nematode control. The results of the articles highlight the potential of the fungus *Pleurotus* spp. and its proteases as a sustainable alternative for the management of important parasites present in agriculture and livestock.

Keywords: Enzymes; Edible mushrooms; Biochemical control; Parasites.

INDICADORES DE IMPACTO

Os resultados obtidos nesta tese apresentam impactos científicos, tecnológicos, econômicos, sociais e ambientais, ao integrar a otimização da produção de proteases por espécies do gênero *Pleurotus*, sua caracterização bioquímica e a avaliação de sua aplicação no controle biológico de patógenos de importância agrícola e veterinária. A pesquisa demonstrou a eficiência da fermentação em estado sólido utilizando o resíduo, farelo de trigo, como meio indutor de proteases, estabelecendo parâmetros que aumentam a produção enzimática e fornecendo informações importantes para futuras aplicações industriais. Os ensaios realizados apresentaram atividade ovicida e larvicida das proteases sobre diferentes patógenos, incluindo nematoides gastrintestinais de ovinos e cestódeos de importância veterinária e em saúde pública, indicando potencial para o desenvolvimento de bioinsumos capazes de reduzir a dependência de anti-helmínticos, minimizar problemas relacionados à resistência parasitária e agregar valor a resíduos agroindustriais por meio da economia circular. Dessa forma, os resultados podem contribuir para a sustentabilidade dos sistemas de produção agrícola e pecuária, beneficiando produtores rurais, empresas do setor de biotecnologia, pesquisadores e consumidores. A pesquisa também está alinhada ao conceito de Saúde Única (One Health), ao propor estratégias de controle que favorecem simultaneamente a saúde animal, a saúde humana e a conservação ambiental, especialmente pela possibilidade de redução do uso de agroquímicos e de seus impactos sobre os ecossistemas. O trabalho apresenta caráter extensionista em potencial, considerando que parte dos experimentos foi realizada com nematoides parasitas gastrointestinais provenientes de condições reais de campo e contou com a colaboração entre a Universidade Federal de Lavras (UFLA), a Universidade Federal de Minas Gerais (UFMG) e a Universidade Vila Velha (UVV), fortalecendo a interação entre instituições de pesquisa e ampliando as perspectivas de transferência tecnológica para o setor produtivo. Os impactos do estudo enquadram-se principalmente nas áreas temáticas de meio ambiente, saúde e tecnologia e produção da política nacional de extensão e estão alinhados aos Objetivos de Desenvolvimento Sustentável (ODS) 2 (Fome Zero e Agricultura Sustentável), ODS 3 (Saúde e Bem-Estar), ODS 9 (Indústria, Inovação e Infraestrutura), ODS 12 (Consumo e Produção Responsáveis) e ODS 15 (Vida Terrestre), evidenciando sua contribuição para o desenvolvimento de soluções biotecnológicas sustentáveis voltadas ao fortalecimento da agropecuária e da bioeconomia.

IMPACT INDICATORS

The results obtained in this thesis have potential scientific, technological, economic, social, and environmental impacts, as they integrate the optimization of protease production by species of the genus *Pleurotus*, their biochemical characterization, and the evaluation of their application in the biological control of parasites of agricultural and veterinary importance. The research demonstrated the feasibility of solid-state fermentation using wheat bran as the fermentation substrate, establishing conditions that enhance protease production while providing essential information for future industrial applications. The experiments demonstrated the ovicidal and larvicidal activities of the proteases against several parasites, including gastrointestinal nematodes of sheep and cestodes of veterinary and public health importance, highlighting their potential for the development of bio-based products capable of reducing dependence on synthetic anthelmintics, mitigating parasite resistance, and adding value to agro-industrial residues within a circular economy framework. Consequently, these findings may contribute to the sustainability of agricultural and livestock production systems, benefiting farmers, biotechnology companies, researchers, and consumers. The research is also aligned with the One Health concept by proposing control strategies that simultaneously promote animal health, human health, and environmental conservation, particularly through the potential reduction in the use of chemical antiparasitic compounds and their associated environmental impacts. Furthermore, the study has potential for extension and technology transfer activities, as part of the experiments was conducted using samples obtained under field conditions through collaborations among the Federal University of Lavras (UFLA), the Federal University of Minas Gerais (UFMG), and Vila Velha University (UVV), strengthening institutional partnerships and expanding the prospects for transferring this technology to the agricultural sector. The impacts of this research fall primarily within the thematic areas of Environment, Health, and Technology and Production, as defined by the Brazilian National Extension Policy, and are aligned with the United Nations Sustainable Development Goals (SDGs), particularly SDG 2 (Zero Hunger), SDG 3 (Good Health and Well-being), SDG 9 (Industry, Innovation and Infrastructure), SDG 12 (Responsible Consumption and Production), and SDG 15 (Life on Land), reinforcing the contribution of this research to the development of sustainable biotechnological solutions that support agriculture and the bioeconomy.

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1 INTRODUÇÃO GERAL

A saúde animal, a humana e a ambiental estão interligadas em um conceito conhecido como Saúde Única, que busca integrar essas três dimensões com o objetivo de promover o bem-estar global (MEDEIROS et al., 2026). Nesse contexto, os nematoides parasitas de plantas assumem grande importância, pois causam perdas significativas na agricultura, comprometendo a segurança alimentar (KANTOR et al., 2024). As perdas anuais globais na produção de culturas alimentícias provocadas por esses fitonematoides são estimadas em cerca de US$ 157 bilhões (BHAT AND RAJA, 2026).

Entre eles, as espécies do gênero *Meloidogyne*, conhecidas como nematoides-das-galhas, destacam-se pela ampla distribuição geográfica e pela variedade de hospedeiros. A espécie *M. incognita* é um endoparasita sedentário capaz de induzir a formação de células gigantes e galhas nas raízes, alterando a absorção de água e nutrientes e comprometendo o desenvolvimento e a produtividade das plantas (KAVITHA et al., 2025; CARPIO et al., 2026). O tomateiro (*Solanum lycopersicum* L.) encontra-se entre as culturas suscetíveis a esse fitonematoide (LEONETTI et al., 2024).

Da mesma forma, nematoides parasitas gastrointestinais, como *Haemonchus* spp., *Trichostrongylus* spp. e *Strongyloides papillosus*, e cestódeos, como *Moniezia* sp. e *Taenia solium*, impactam negativamente a pecuária, afetando o desenvolvimento animal e causando prejuízos econômicos (BOUGHTON et al., 2024; ALMEIDA-CAICEDO et al., 2023; MINANI et al., 2022). Além disso, *T. solium* possui importância zoonótica, podendo causar doenças em seres humanos, como a cisticercose, uma doença grave que pode afetar o sistema nervoso central. Por isso, reforça-se a necessidade de estratégias eficazes e sustentáveis para o controle desses parasitas (ARAÚJO et al., 2024; MØLLER et al., 2024; RIBEIRO et al., 2023; ALI et al., 2023).

O controle químico desses parasitas é uma abordagem amplamente utilizada. No entanto, os produtos químicos podem causar sérios problemas ambientais no solo, na água ou no ar, devido à sua toxicidade (AYAZ et al., 2024; ZHOU et al., 2024). O uso contínuo ou inadequado desses produtos pode favorecer a seleção de indivíduos resistentes e ocasionar efeitos indesejáveis sobre organismos não alvo (BOWDRIDGE et al, 2025). Por outro lado, o controle biológico tem sido descrito como uma estratégia para o manejo de pragas e utiliza organismos vivos para controlá-las (ZHOU et al., 2024). Esses agentes de controle biológico apresentam vantagens significativas em relação aos produtos químicos, incluindo maior especificidade ao alvo e menor persistência no ambiente e ausência de seleção de indivíduos

resistentes. Muitos desses agentes atuam como inimigos naturais dos organismos-alvo (FERREIRA; SOARES, 2023).

Outra alternativa promissora para o controle de parasitas é o uso de enzimas. Nesse sentido, as proteases (EC 3.4) são as principais enzimas envolvidas nesse processo. Essa classe nas estruturas dos parasitas (SOARES et al., 2023). Essa estratégia consiste em uma forma de controle bioquímico que utiliza macromoléculas de origem vegetal ou microbiana (SOARES; WANDERLEY; COSTA, 2019).

O gênero *Pleurotus* possui várias espécies de cogumelos reconhecidas por sua atividade nematófaga (BELLO et al., 2025). Esses fungos produzem metabólitos com ação tóxica sobre os nematoides, incluindo o ácido trans-2-decenedioico, além de diferentes compostos aromáticos, ácidos graxos e terpenos (GÓMEZ-RODRÍGUEZ et al., 2022). Esses metabólitos podem promover a imobilização ou a morte dos nematoides, facilitando, posteriormente, a colonização pelas hifas e a ação de enzimas hidrolíticas (NYANGWIRE et al., 2024). Os fungos e as enzimas secretadas por *Pleurotus* podem ser utilizados de forma conjunta ou isolada no controle biológico e bioquímico de parasitas (SILVA et al., 2024).

Além disso, *Pleurotus* spp. são cogumelos comestíveis de excelente valor nutricional e podem ser cultivados em resíduos agrícolas, como polpa de café, bagaço de cana-de-açúcar, farelo de trigo e outros materiais lignocelulósicos (RODRÍGUEZ-BARRERA et al., 2021). Essa prática não apenas possibilita o reaproveitamento de subprodutos agrícolas, mas também promove sua reinserção na economia, alinhando-se aos princípios da economia circular (VIRIATO et al., 2024).

Dessa forma, surge a necessidade de otimizar a produção de enzimas por *Pleurotus* spp. e avaliar tanto a atividade antiparasitária dos fungos quanto a de suas enzimas proteolíticas sobre ovos e juvenis de nematoides parasitas de plantas, nematoides parasitas gastrointestinais e ovos de *Taenia solium* e *Moniezia* sp. Além desses organismos parasitários, foi empregado nesta tese um nematoide de vida livre, *Panagrellus* sp., como organismo-modelo. Por fim, faz-se necessário investigar a compatibilidade das proteases produzidas por *Pleurotus* com uma protease de origem vegetal, como a papaína, visando ampliar as perspectivas para o desenvolvimento de formulações enzimáticas mais eficientes para o controle bioquímico de parasitas de interesse agropecuário.

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2 CAPÍTULO 1

ARTIGO 1:

Proteases from *Pleurotus* spp.: Properties, Production and Biotechnological Applications

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COLABORADORES:

Adriane Toledo da Silva [1], Liliana Aguilar-Marcelino [2]*, Amanda do Carmo Alves [1], Débora Castro Toledo de Souza [1], Ana Carolina Silva [1], Jhennifer Cristina de Souza Alves [1], Yanick Leontino Langa [1], Elias Honorato Gomes [1] and Filippe Elias de Freitas Soares [1]

1 Departamento de Química, Laboratório de Biotecnologia e Bioquímica Aplicada, Universidade Federal de Lavras

2 Centro Nacional de Investigación Disciplinaria en Salud Animal e Inocuidad (CENID-SAI), Instituto Nacional de Investigaciones Forestales, Agrícolas y Pecuarias (INIFAP). Carretera Federal Cuernavaca-Cuautla No. 8534, Colonia Progreso, C.P. 62390, Jiutepec, Morelos, México

*Corresponding author – aguilar.liliana@inifap.gob.mx

Review

Proteases from *Pleurotus* spp.: Properties, Production and Biotechnological Applications

Adriane Toledo da Silva [1], Liliana Aguilar-Marcelino [2]*, Amanda do Carmo Alves [1], Débora Castro Toledo de Souza [1], Ana Carolina Silva [1], Jhennifer Cristina de Souza Alves [1], Yanick Leontino Langa [1], Elias Honorato Gomes [1] and Filippe Elias de Freitas Soares [1]

1 Laboratório de Biotecnologia e Bioquímica Aplicada, Departamento de Química, Universidade Federal de Lavras, Lavras – MG, Brasil
2 Centro Nacional de Investigación Disciplinaria en Salud Animal e Inocuidad (CENID-SAI), Instituto Nacional de Investigaciones Forestales, Agrícolas y Pecuarias (INIFAP). Carretera Federal Cuernavaca-Cuautla No. 8534, Colonia Progreso, C.P. 62390, Jiutepec, Morelos, México
* Correspondence: author – aguilar.liliana@inifap.gob.mx

Abstract

Proteases (EC 3.4) are hydrolytic enzymes widely used in biotechnological processes, representing about 60 to 70% of the global industrial enzyme market. Edible mushrooms of the genus *Pleurotus* stand out as excellent producers of these enzymes, in addition to exhibiting high nutritional value and medicinal properties. The proteases produced by these species exhibit broad adaptability to different experimental conditions, including variations in optimal pH and temperature, as well as distinct sensitivities to inhibitors. The production of these enzymes can be intensified by solid-state fermentation (SSF) using low-cost agro-industrial substrates, such as wheat bran, which favors sustainable applications aligned with the circular economy. Parameters such as carbon/nitrogen (C/N) ratio, medium pH, cultivation time, and inoculum age directly influence enzyme productivity. Proteases from *Pleurotus* spp. show high potential in the biochemical control of parasites such as *Meloidogyne incognita*, *Haemonchus* spp., *Taenia solium*, and *Moniezia* sp., catalyzing the degradation of the cuticle or eggshell. Other biotechnological applications include milk coagulation, thrombolytic therapies, keratin bioconversion, increased protein digestibility, and use as additives in the food, detergent, and pharmaceutical industries.

Keywords: edible mushroom; biocontrol; fungi; omics

1. Introduction

Proteases (EC 3.4) are a type of enzyme that helps break down peptide bonds, leading to the formation of peptides and amino acids [1, 2, 3]. All forms of life contain proteins in their constitution, which play essential roles in fundamental physiological processes such as immune defense, cell growth and death, sporulation, blood coagulation, enzymatic modification, and regulation of gene expression [4,5,6]. These functions confer great biological importance to proteins and, consequently, to proteases. In addition to their physiological relevance, these enzymes occupy a significant share of the industrial market, accounting for approximately 60% to 70% of the enzymes sold worldwide [7, 8, 9].

Fungi stand out as important microorganisms for protease production [8]. Within this group, edible mushrooms, especially those belonging to the genus *Pleurotus* (phylum Basidiomycota), have gained prominence [10]. The genus *Pleurotus* comprises more than 40 species [4], among which the edible mushrooms *P. ostreatus* (oyster) and *P. eryngii* (king oyster or shimeji) stand out, being widely appreciated and consumed. Globally, *P. ostreatus* is the second most produced mushroom [10, 11]. Several factors justify its importance, including medicinal properties, reduced cultivation time

compared to other edible mushroom genera, and the ability to colonize and degrade various types of lignocellulosic waste, which favors practices associated with the circular economy [7, 9, 12].

Proteases of *Pleurotus* spp. have been used promisingly in the biochemical control of different parasites of importance in the context of One Health, such as *Taenia solium*, *Moniezia* sp. [13], *Meloidogyne incognita* [14], *Trichostrongylus* spp., and *Strongyloides papillosus* [15]. They act to catalyze the degradation of proteins present in the structure of the parasites [13]. In addition, these enzymes have potential applications in milk coagulation for cheese production, thrombolytic therapy for the treatment and prevention of thrombosis, and various industrial processes in the detergent, food, and pharmaceutical sectors [3, 16, 17].

Given the favorable characteristics of proteases from the genus *Pleurotus* and the growing industrial demand for biodegradable methods with low operating costs [18], research involving these fungi has gained increased attention, even though the genera *Aspergillus* and *Penicillium* have historically been the most widely used for protease production [4]. In this context, this review aims to compile up-to-date information on the classification and biochemical characterization of proteases produced by different *Pleurotus* species, as well as discuss their various biotechnological applications.

2. Classification and Biochemical Characterization of *Pleurotus* Proteases

The genus *Pleurotus* encompasses a large number of species of protease-producing edible mushrooms [4] such as *P. ostreatus* [11], *P. eryngii* [1], *P. citrinopileatus* [19], *P. pulmonarius* [12], *P. sajor-caju* [18], *P. djamor* [20], *P. albidus* [21], and *P. tuber-regium* [22]. Among these species, the most investigated is *P. ostreatus* [4], due to several factors, such as its high nutritional and medicinal value, excellent yield, ease of large-scale cultivation, and ability to produce a variety of enzymes [4,11].

Among the enzymes produced by species of the genus *Pleurotus*, proteases stand out as one of the most relevant [4,12]. These enzymes can be classified based on their mechanism of action, dividing them into exopeptidases, which promote the catalysis of peptide bond cleavage at the N- or C-terminals, and endopeptidases, responsible for the catalysis of internal bond cleavage in the polypeptide chain [1, 23]. In addition, another important classification criterion is the catalytic mechanism involved in hydrolysis, which can be determined by analyzing the amino acid residues or cofactors present at the enzyme's active site [24, 25].

Additionally, proteases can also be classified into families, according to the MEROPS database, which organizes these enzymes based on structural, functional, and evolutionary characteristics. Each family receives an alphanumeric designation, such as S8 (serine proteases) or M36 (metalloproteases/fungalisins) [26].

The most common class of proteases produced by *Pleurotus* spp. is that of serine proteases, whose catalytic triad is composed of aspartate, histidine, and serine residues [9]. During the peptide bond cleavage process, the serine residue acts as a nucleophile, initiating the catalytic reaction. In the industrial context, this type of protease is highly relevant, which explains its great demand on a global scale [21,9]. In MEROPS, these enzymes are mainly assigned to the S8 family. Their activity can be affected by certain inhibitors, such as diisopropyl fluorophosphate (DFP) [27], 3,4-dichloroisocoumarin (DCI), 4-(2-aminoethyl) benzenesulfonyl fluoride (AEBSF), leupeptin, aprotinin, and PMSF (phenylmethylsulfonyl fluoride), the latter being the most cited inhibitor in the literature [2].

On the other hand, although to a lesser extent, aspartic proteases are also produced by *Pleurotus* spp. [28]. These enzymes share several biochemical and structural features, such as sequence similarity, predominance of beta-sheet secondary structures, and the presence of aspartate residues at the active site, which are responsible for catalytic activity [29]. Due to their optimal performance under acidic pH conditions, these enzymes are classified as acid proteases [12]. In MEROPS, they mainly +6 correspond to the A1 family. Inhibitors such as diazoacetylnorleucine (DAN) methyl ester, 1,2-epoxy-3-(p-nitrophenoxy) propane (EPNP), and pepstatin A affect the activity of aspartic proteases, with pepstatin A being the best known and most widely used [2].

Another group of proteases present in the genus Pleurotus is the cysteine proteases, also known as thiol proteases. The active site of these enzymes contains three amino acids that form the catalytic triad Cys/His/Asn (or Asp), whose residues act in a coordinated manner in the hydrolysis of the peptide bond [30]. Although they are not the most frequently found proteases, studies indicate their occurrence in *P. ostreatus*, *P. albidus*, and *P. sajor-caju* [4,21]. In MEROPS, they are grouped into families such as C1. Their activity is inhibited by thiol group-chelating compounds, such as iodoacetic acid [2].

Metalloproteases are also present in *Pleurotus* species, such as *P. ostreatus* and *P. eryngii* [17,31]. The enzymatic catalysis of these proteases requires the coordination of a metal ion at the active site. During peptide bond hydrolysis, the water molecule, acting as a nucleophile, is activated by the metal ion [32]. In MEROPS, metalloproteases of the M36 family, also known as fungalisins, are particularly noteworthy and are frequently found in basidiomycetes. The activity of these enzymes is inhibited by metal-chelating agents, such as EDTA (ethylenediaminetetraacetic acid) and 1,10-phenanthroline [2,29].

The presence of different classes of proteases may be associated with the different morphological stages of edible mushrooms. In the study by Genier et al. (2015), for example, the authors suggested that the protease produced by *P. ostreatus* is a serine protease, based on the total inhibition of enzymatic activity observed in the presence of a specific inhibitor of this class [33]. On the other hand, Choi and Shin (1998), using the fruiting body as the enzyme source in the purification and characterization stage, identified a cysteine protease produced by *P. ostreatus* [34].

In genomic terms, the genome of the PC9_AS strain of *P. ostreatus* was annotated with approximately 11,875 genes, a number similar to that found in strains PC15 and PC9_JGI, which have 12,330 and 12,206 genes, respectively [35]. The annotation includes, among other elements, protease-encoding genes classified by the MEROPS database, suggesting considerable diversity of these enzymes in the genome. Transcriptomic studies reinforce this enzymatic diversity: Alfaro et al. (2016) identified at least 18 peptidase families, including serine, cysteine, aspartic, and metalloproteases, with emphasis on the S8 (serine proteases) and M36 (metalloproteases) families, which showed high expression under different culture conditions [36]. In addition, Faraco et al. (2005) characterized a new subfamily of serine proteases in *P. ostreatus*, describing the structure and function of an extracellular protease encoded by the posl gene, belonging to the S8 family, confirming its relevant role in the degradation of lignocellulosic substrates [37]. The review by Inácio et al. (2005) highlights that the genus *Pleurotus* possesses a variety of extracellular proteases, reflecting an adaptive strategy that contributes to its efficiency in decomposing organic matter, especially in environments rich in lignin and cellulose [4]. However, despite the identification of multiple peptidase families and the functional annotation of the genome, to date there are no studies that present, in a consolidated manner, the total number of protease-encoding genes in the genus *Pleurotus*, which remains a gap in the literature.

Table 1 presents the main biochemical properties of proteases produced by various *Pleurotus* species, including molecular weight, optimal pH and temperature, sensitivity to inhibitors, and response to metal ions. These parameters can vary significantly between and within species due to differences in the media used to induce enzyme production, which highlights the functional diversity of fungal proteases.

Table 1. Biochemical properties of proteases from species of the genus *Pleurotus*.

Species	Molecular weight (kDa)	Optimal pH	Optimal temperature (°C)	Km	Vmáx	Sensitivity to inhibitors	Influence of metal ions on enzymatic activity
P. eryngii	11,5 [38] 14 [29]	5,0 [38] 6,5 [1]	40 [27]	0.18 mM [10]	53.5 U/mL [10]	Pepstatin A inhibits pleureryn; PMSF does not affect it [38] The fibrinolytic enzyme was completely inhibited by PMSF [27]	-

P. citrinopileatus	28 [19]	10 [19]	50 [19]	3,44 mg/mL [19]	0,139 mg/mL.min [19]	PMSF completely inhibited, EDTA did not inhibit, Pepstatin A moderately inhibited [19]	Activation by K^+, Li^+, and Mg^{2+}; inhibition by Al^{3+}, Cu^{2+}, Hg^{2+}, Zn^{2+}, Pb^{2+}, Co^{2+}, Mn^{2+}, and Ca^{2+} [19]
P. ostreatus	18,2 – 97 (24, 29, 34, 39, 28, 40, 41)	6,5–9 [20, 33, 39,42]	35–75 [33, 34, 41,42]	-	-	PMSF completely inhibited [2, 32, 39], Pepstatin A inhibits acid protease [2], *P. ostreatus* subtilisin-like protease was not inhibited by Pepstatin A [39], EDTA inhibited it completely [33], and protease was inhibited by EDTA only for a certain period of time to 74.3% [2]	Ca^{2+} has a positive effect on activity [39]. Cu^{2+}, Al^{3+}, and Hg^{2+} cause inhibition. Activity was restored by Zn^{2+} or Co^{2+} [19]
P. pulmonarius	16 [43]	5,5 [12]	45 [12]	0,61 mg/mL	1,79 mM/min	Pepstatin A (56% inhibition), PMSF (63.4% inhibition), EDTA (81.0% maximum inhibition) [12]	Inhibition by Mn^{2+}, Ba^{2+}, Fe^{2+}, and Ca^{2+}. Slight increase by Mg^{2+} [12]
P. albidus	36 [5]	7,0 [5] 5,0 [23]	40-50 [5,23]	-	-	PMSF (30% inhibition) [23]. Pepstatin A and EDTA did not inhibit [21]	Mn^{2+}, Cu^{2+}, Na^+, Mg^{2+}, K^+, Zn^{2+}, and Ca^{2+} cause slight inhibition. Fe^{2+} increased activity [21, 23]
P. ostreatoroseus	—	7,0 [44]	40 [44]	-	-	PMSF (94% inhibition). EDTA (87% inhibition) [21]	Cu^{2+}, Zn^{2+}, and Mn^{2+} cause inhibition, while Fe^{2+}, Mg^{2+}, Ca^{2+}, K^+, and Na^+ cause slight inhibition [21]
P. sajor-caju	48 e 65 [18, 45]	8,0 [45]	60 [45]	0,275 mg/mL	79 μmol/mg/min	PMSF completely inhibited, EDTA slightly inhibited, and pepstatin A did not inhibit [18]	Cd^{2+}, Ni^{2+}, Hg^{2+} were inhibited completely. Co^{2+}, Ba^{2+}, Zn^{2+} inhibited partially. Activation by Ca^{2+}, Mg^{2+}, Mn^{2+}, and Fe^{2+} increased activity [18]
P. djamor	75–100 [46]	5,0 e 8,0 [9, 20]	50 [20]	-	-	PMSF (45% inhibition) and EDTA (69% inhibition) [9]	-

When analyzing Table 1, the wide adaptability of *Pleurotus* spp. proteases to environmental conditions can be observed. These enzymes show significant variations in their optimal pH and temperature ranges [2, 23]. In addition, the use of specific inhibitors for different classes of proteases, such as PMSF, pepstatin A, and EDTA, has revealed the presence of multiple classes of proteases in the same species [2,5]. The relevance of metal ions in enzymatic catalysis further reinforces the importance of detailed characterization of these enzymes, especially for their application in biotechnological processes involving different matrices and physicochemical conditions. [5, 12].

3. Physiological aspects of protease production by *Pleurotus* spp.

The presence of different classes of proteases may be associated with the different morphological stages of the edible mushrooms. In the study by Genier et al. (2015), for example, the authors suggested that the protease produced by *P. ostreatus* is a serine pro-tease, based on the total inhibition of enzymatic activity observed in the presence of a spe-cific inhibitor of this class [33]. On the other hand, Choi and Shin (1998), using the fruiting body as an enzyme source in the purification and characterization stage, identified a cys-teine protease produced by *P. ostreatus* [34].

The age of the inoculum is a critical and easily controlled factor that directly impacts protease production yield [4]. Previous studies indicate that a young inoculum in an active growth phase results in significantly higher enzyme production [21]. The study by Martim et al. (2017) using *P. albidus* found maximum activity (80.33 U/mL) with a five-day-old in-oculum. Subsequently, the authors observed a gradual decrease in activity with increasing inoculum age, with reductions of 28.57%, 47.40%, and 77.39% in proteolytic activities for inocula aged 8, 12, and 20 days, respectively. [21]

The cultivation time determines the peak production of both biomass and enzymes, varying significantly among *Pleurotus* spp. species (*P. sajor-caju* at 4 days, *P. djamor* at 5 days, and *P. pulmonarius* at 10 days) [4, 45]. Exceeding the ideal time results in a decrease in enzymatic activity, possibly due to enzymatic degradation or toxin accumulation [7, 24].

A key variable that controls both microbial growth and protease production for *Pleu-rotus* spp. is the C/N ratio in the substrate [45]. Adequate amounts of carbon, particularly from lignocellulosic sources such as wheat, stimulate the development of the fungus, as proteases production is often inhibited by a lack of carbon and nitrogen, forcing the fun-gus to secrete these enzymes to obtain nutrients from the medium. On the other hand, an excess of nitrogen can be toxic and inhibit mycelial growth [7].

In addition to hydrolyzing proteins, *Pleurotus* spp. proteases play an important role in regulating other enzymes produced by the fungus. It has been demonstrated that the extracellular acid and neutral proteases produced by P. ostreatus modulate the activity of laccase isoforms by degrading enzymes such as POXA1b via the serine protease PoSl [2, 18]. Conversely, when these proteases are inhibited by inhibitors such as PMSF and pepstatin A, laccase activity increases by a factor of 1.35 [2, 37].

In contrast, alkaline proteases have been shown to have a low or even positive effect on *P. ostreatus* activity. This indicates that laccase is not sensitive to this type of enzyme and can therefore act as an activator of laccase activity [4, 10]. The literature also highlights possible synergistic or competitive interactions between proteases and laccases influenced by substrate composition, revealing a highly adaptive enzyme system [2].

At the physiological level, these enzymes act in spore germination, sporulation, and substrate degradation, being fundamental for the survival of the fungus in lignocellulosic environments [2,4]. The co-expression of proteases and laccases produced by *P. sapidus* suggests the presence of integrated enzyme systems, although these are still poorly understood [11].

4. Production and Optimization of *Pleurotus* Proteases

The production of enzymes by mushrooms of the genus *Pleurotus* mainly uses two different strategies: solid-state fermentation (SSF) and submerged fermentation (SmF) [7,24]. These fungi are significant producers of various enzymes, among which proteases stand out for their extensive industrial applications. Therefore, selecting an appropriate cultivation method is essential to increase enzyme production and economic viability [9, 19].

Solid-state fermentation (SSF) mimics the natural habitat of many fungi, cultivating microorganisms in isolated or low-moisture substrates [47]. Because it uses less water and energy and produces less harmful waste, this process is believed to be more cost-effective [48]. On the other hand, submerged fermentation (SmF) (Table 2) involves the cultivation of microorganisms in a liquid medium. Immersing the fungus provides greater control over culture parameters such as pH, temperature,

oxygen levels, and humidity, in addition to simplifying the purification of extracellular enzymes and large-scale production [21, 49, 50].

Table 2. Submerged fermentation (SmF) for protease production by *Pleurotus* spp. species.

AGRO-INDUSTRIAL RESIDUES	*PLEUROTUS* SPECIES	PROTEOLYTIC ACTIVITY (U/mL)	STUDY
Glucose, yeast extract	*P. ostreatus*	90.0	[33]
Glucose, yeast Extract, peptone	*P. albidus*	73.39	[21]
Potato dextrose broth	*P. sajor-caju*	10.5	[18]
Yeast Malt	*P. ostreatus*	1015.1	[17]
Yeast Malt	*P. eryngii*	1552.8	[17]
Sabouraud dextrose, yeast extract	*P. ostreatus*	1512.0	[17]
Sabouraud dextrose, yeast extract	*P. eryngii*	1086.4	[17]
Glucose, peptone, yeast extract	*P. ostreatoroseus*	1.361.73	[51]
Malt, glucose, peptone, yeast extract	*P. ostreatoroseus*	262.02	[51]
Malt	*P. ostreatoroseus*	382.32	[51]

The choice between SSF and SmF requires a strategic assessment of the various possibilities and production interests. On the one hand, SSF is commonly chosen because it generates a more concentrated enzyme product, which facilitates and reduces the costs of the extraction and purification phases, making it attractive in some biocatalysis processes [12]. However, despite higher initial costs, SmF offers better process control and greater scalability, essential factors for consistent industrial production. Both approaches are being actively investigated to maximize protein yield for the growing biotechnology market [2, 20].

In general, the success of this species in bioprocesses is due to its ability to grow easily in tropical and subtropical regions. *Pleurotus* spp. species are especially suitable for SSF because they can thrive on a variety of lignocellulosic agricultural wastes and are mainly capable of mimicking their natural growth environment, as shown in Table 3 [52].

Table 3. Lignocellulosic and agroindustrial residues used as alternative substrates for protease production by *Pleurotus* spp. species.

AGRO-INDUSTRIAL RESIDUES	*PLEUROTUS* SPECIES	PROTEOLYTIC ACTIVITY (U/mL)	STUDY
Wheat Bran	*P. albidus*	5.029	[5]
Wheat Bran	*P. djamor*	31.61	[13]
Wheat Bran	*P. pulmonarius*	76.3	[12]
Wheat Bran	*P. sajor-caju*	22	[45]
wheat grains	*P. ostreatus* (SB)	5.0	[20]
wheat grains	*P. ostreatus (Pearl)*	4.0	[20]

Oat bran	*P. pulmonarius*	73.0	[12]
Corn Cob	*P. eryngii*	53.5	[28]
Corn Flour	*P. sajor-caju*	45	[45]
Corn pomace	*P. pulmonarius*	94.0	[12]

The proteases produced by fungi of the *Pleurotus* genus show highly competitive enzymatic activity, with values that often exceed those of other fungi in specific applications, although direct comparison requires methodological caution [24]. An example of this is the fibrinolytic activity of the *P. eryngii* species, which showed larger activity halos (63 mm^2) than *Agaricus blazei* (35 mm^2) and a specific activity of 226.47 U/mL, higher than that of *P. ostreatus* (71.5 U/mL) [6, 17]. Even more significantly, the serine protease produced by *P. sajor-caju* showed a catalytic efficiency up to 8.48 times greater than that of *Aspergillus oryzae* [18]. On the other hand, in some cases it is possible to observe that genera that belong to the Ascomycota phylum excelled in proteolytic activity, such as *Duddingtonia flagrans* (56.34 U/mL) [53], *Beauveria bassiana* ESALQ PL63, and *Metarhizium anisopliae* ESALQ E9 (36 U/mg and 52 U/mg), respectively. [54]. During the milk curdling process, the gross activity of *P. albidus* (153 U/mL) remained lower than that of *Aspergillus* sp. (240 U/mL) [5]. It is important to note that these values are dependent on the substrate and test conditions, but the consolidated evidence positions *Pleurotus* as a versatile and potent source of proteases with high biotechnological potential [4].

The efficiency of the *Pleurotus* genus in degrading lignocellulosic substrates is what underpins its use in circular economy applications, such as the production of proteases from agro-industrial waste [55, 56]. As decomposition is necessary for its growth, the fungus actively seeks out these materials, which are rich in cellulose, hemicellulose, and lignin [57]. These fungi are able to carry out this process because they produce a complex of ligninolytic and hydrolytic enzymes, including proteases, which act synergistically to catalyze the breakdown of vegetative cell wall polymers [52, 58]. Thus, while the fungus performs its ecological function of recycling nutrients, it also produces high-value biotechnological enzymes, converting waste into a product [3, 59].

For the enzymatic production process to transcend the laboratory scale and become an industrially viable process, it is important to rigorously optimize the cultivation parameters [51]. Variables such as pH, temperature, incubation time, agitation, and the nutritional composition of the substrate, particularly the carbon/nitrogen (C/N) ratio, interact synergistically to modulate the fungus's metabolism and directly affect its production process. Precise control of these variables is what determines the commercial success of the bioprocess, maximizing productivity, reducing operating costs, and ensuring the acquisition of enzymes with the desired activity and stability [48].

The pH of the culture medium influences the activity, production, and stability of *Pleurotus* spp. proteases [23]. Each species and even each type of protease may have a distinct optimum pH (e.g., pH 6.5 for *P. eryngii*, pH 9.0 for *P. ostreatus* alkaline) [1, 33]. The pH of the medium is also dynamic, being influenced by factors such as the C/N ratio, and its optimization is vital not only for production but also for specific applications, such as milk coagulation in cheese making [23].

5. Applications of *Pleurotus* proteases

5.1. Nematicide applications and biological control

In addition to their importance in human nutrition and biotechnological processes, mushrooms of the *Pleurotus* genus have attracted growing interest for their potential in the biological control of animal parasites such as *Moniezia* sp., *T. solium*, and *Haemonchus* spp. and plant parasites such as *M. incognita* [13, 14, 15, 46]. The use of *Pleurotus* spp. as biocontrol agents represents a promising alternative to reduce dependence on chemical pesticides, contributing to more sustainable and environmentally friendly agricultural practices [11].

These fungal species act through different mechanisms to control parasites, one of which is the production of toxins, such as trans-2-decenedioic acid, capable of paralyzing target organisms. Once

paralyzed, the parasites become entangled in the fungal hyphae and exhibit morphological deformities, such as a reduction in head volume [60, 61].

Another important mechanism involves the secretion of extracellular proteases. The cuticle of nematodes, composed predominantly of proteins, functions as a protective barrier. Proteases produced by *Pleurotus* spp. act directly on this structure, catalyzing the hydrolysis of cuticular proteins, leading to its degradation and, consequently, to the death of the parasite [14] (Figure 1).

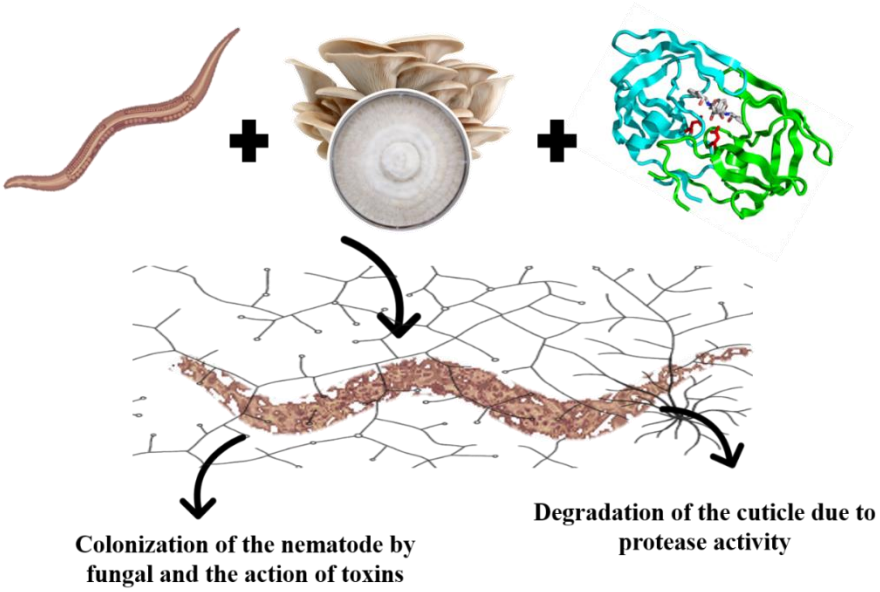

Figure 1. Mechanism of nematophagy of edible mushroom.

In vitro studies have used free-living nematodes, such as *Panagrellus* sp., as models to evaluate the efficacy of compounds produced by *Pleurotus* spp. Genier et al. (2015) evaluated the predatory activity of *P. ostreatus* (strain PLO 06) on *Panagrellus* sp. larvae, observing a 65.6% reduction in the number of larvae on the first day, 77.4% on the second day, and 95.2% on the third day. In the same study, in another trial, only the extracellular proteases from the same isolate were used, resulting in a 42% reduction in the number of larvae. This result indicates that the proteases produced by *P. ostreatus* (90 U/mL) are essential for the cuticle degradation process, enhancing the nematicidal effect [33].

The combined action of toxins that paralyze nematodes and hydrolytic enzymes contributes to the biocidal effect promoted by species of the genus *Pleurotus.* Plant parasitic nematodes cause great damage to agriculture worldwide, directly affecting crop yields and requiring more effective control strategies [14]. In this context, Sufiate et al. (2017) demonstrated that *P. eryngii* has nematicidal activity on eggs and juveniles of *M. javanica*, with a reduction of more than 50%, attributed to the production of proteases (32.74 U/mL) and chitinases (3.57 U/mL) [62].

Tests conducted with animal parasitic nematodes also demonstrate the nematicidal potential of *Pleurotus* spp. proteases. Silva et al. (2025) evaluated the effect of the cell-free crude extract, rich in *P. djamor* proteases (proteolytic activity of 7.5 U/mL and specific activity of 30 U/mL), on ruminant coprocultures and observed a 35% reduction in the number of *Haemonchus* spp. and *Trichostrongylus* spp. larvae, a result considered promising, especially as it was obtained in a fecal environment [46].

In addition to nematodes, in vitro tests on *T. solium* and *Moniezia* sp. eggs demonstrated the potential of P. djamor proteases (31.61 U/mL) in controlling these parasites. Silva et al. (2025) reported reductions of 33.44% in the hatching of *T. solium* eggs and 45.43% in *Moniezia* sp. eggs, highlighting the role of proteases in the degradation of eggshells [13].

The synergistic action between toxins and enzymes intensifies the observed effects. Trans-2-decenedioic acid, for example, initially acts by paralyzing the parasites, compromising their ability to

respond and making them more susceptible to enzymatic action [20]. In turn, the degradation of the cuticle promoted by proteases facilitates toxin penetration, establishing an effective cycle of action. This synergy gives *Pleurotus* spp. a significant advantage as biocontrol agents [33]

The use of *Pleurotus* spp. or its cell-free crude extracts has several ecological advantages. However, further advances are still needed in the standardization of fermentation processes, purification of bioactive compounds, and more in-depth field studies to assess enzyme stability [11]. *Pleurotus* spp. and their proteases thus proves to be a promising biotechnological tool in control different parasites. The combined action of toxic metabolites and extracellular proteases confers high efficacy in the immobilization and degradation of these organisms. Case studies with different species of the genus [33, 62] reinforce this potential, indicating the possibility of developing effective and sustainable bio-inputs for modern agriculture.

5.2. Food

Cheese production reflects the cultural and culinary traditions of each country and region. In 2019, the global cheese market was valued at approximately US$114.1 billion. Europe is the leading producer, accounting for nearly half of the total market value. In terms of consumption, the United States ranks first, followed by Germany and France. It is estimated that by the end of 2030, global cheese production will reach 27 million tons, underscoring the relevance of cheese as a key product in the food sector [63].

An essential step in cheese production is milk coagulation. This process occurs through the hydrolysis of peptide bonds in κ-casein, a protein present in milk, which leads to micelle destabilization and, consequently, the precipitation of casein [42]. One of the main methods used for inducing milk coagulation involves the action of hydrolytic enzymes, with chymosin (EC 3.4.23.4) being the most widely employed. This protease is considered the standard enzyme for milk coagulation in the production of various types of cheese due to characteristics such as high coagulating activity and specificity for cleaving the Phe105–Met106 peptide bond in κ-casein [23].

Renin, a complex of aspartic endopeptidases, is the main source of proteases used in cheese production. However, the use of raw materials of animal origin, such as bovine stomachs, has prompted the search for alternative sources of milk-coagulating enzymes. Species of the *Pleurotus* genus have a remarkable ability to adapt to different environments, since they are able to colonize and degrade a wide variety of lignocellulosic residues, in addition to growing in different temperature ranges and geographical regions [33, 18]. It is one of the most widely distributed genera of edible mushrooms in the world, and its cultivation is facilitated by its low nutritional requirements [4, 18]. In addition, they produce high levels of various enzymes, particularly proteases that are essential for the hydrolysis of milk proteins, making them promising alternatives as sources of enzymes for cheese production [18, 21, 42].

Studies have shown that one species of this genus, *P. albidus*, native to the Amazon, is capable of synthesizing milk-coagulating proteases in liquid culture media. Martim et al. (2021) demonstrated that the proteases produced by *P. albidus* (DPUA 1692), when cultivated on açaí seeds supplemented with rice bran, were effective in the production of *Minas frescal* cheese. Fermentation time and temperature, as well as the concentration of certain metal ions such as calcium, influenced their coagulating activity [23].

A previous study by Martim et al. (2017) also reported that *P. albidus* (DPUA 1692) produces milk-coagulating enzymes with optimal activity at 60°C and pH 6 [21]. Nolli et al. (2022) observed that the crude extract of *P. albidus* with an activity value of 153 U/mL was sufficient to coagulate milk [5].

In addition, another edible mushroom species that has shown the ability to produce milk-coagulating proteases is *P. djamor*. Research conducted by Silva et al. (2025) reported the production of these enzymes by *P. djamor* PLO13 cultivated in wheat bran, identifying them as serine proteases. These enzymes were capable of coagulating both whole and skimmed pasteurized milk, even at room temperature and in the presence or absence of calcium. According to the authors, 1.875 mg/mL of

protease-containing protein in the crude extract of *P. djamor* is required to coagulate milk at a temperature of 50°C in 30 minutes for pasteurized whole milk and in 45 minutes for reconstituted skimmed milk [9].

5.3. Biomedical

According to an article published by Mensah et al. (2023), the global death toll from cardiovascular disease rose from 12.4 million in 1990 to 19.8 million in 2022, reflecting the growth and aging of the world's population, as well as the influence of preventable risk factors of a metabolic, behavioral, and environmental nature [64]. In addition, although the available drugs are safe, they are still expensive [65]. In this context, there is evidence that enzymes produced by microorganisms, such as fungi, may be useful in controlling cardiovascular diseases due to their ability to produce fibrinolytic enzymes [24].

Fibrinolytic enzymes are proteases capable of promoting the catalysis of the degradation of the fibrin mesh, the main protein component present in blood clots. Among the species capable of producing fibrinolytic enzymes are mushrooms of the genus *Pleurotus*, such as *P. ferulae*, *P. ostreatus*, *P. eryngii*, and *P. pulmonarius* (Table 4). The fibrinolytic proteases produced by these fungi have been identified as SPPs, metalloproteases, serine proteases, and serine metalloproteases, showing therapeutic potential against thrombosis [17, 4].

Table 4. Studies of fungi of the genus *Pleurotus* that showed fibrinolytic enzyme production and activity.

PLEUROTUS **SPECIES**	**CULTIVATION METHOD**	**SPECIFIC ACTIVITY**	**FIBRINOLYTIC ACTIVITY**	**EVALUATION METHOD**	**STUDY**
P. ostreatus	Fermentation in submerged culture	1.199,75 U/mg	-	Fibrin plate method	[49]
P. ostreatus	Soybean Bran + Yeast Extract	-	100.14 U/mL	Formation of an artificial thrombus	[17]
P. ostreatus	Yeast Extract Malt broth	-	71.5 ± 0.56 U/mL	Formation of an artificial thrombus	[17]
P. eryngii	Yeast Extract Malt broth	-	226.47 U/mL	Formation of an artificial thrombus	[17]
P. eryngii	Soybean Bran + Yeast Extract	-	71.49 U/mL	Formation of an artificial thrombus	[17]
P. albidus	Solid state fermentation (Black-eyed peas)	850 U/mg	181.11 U/mL	Fibrin plate method	[65]

P. albidus	Solid state fermentation(Thorn yam)	310 U/mg	156.01 U/mL	Fibrin plate method	[65]
P. albidus	Solid state fermentation (Wheat grain)	180 U/mg	128.40 U/mL	Fibrin plate method	[65]
P. ostreatus	Malt extract agar	6.45 mm^2	-	Fibrin plate method	[3]
P. ferulae	-	1.253,33 U/mg	376 U/ mL	Fibrin plate method	[31]

5.4. Industrial

Keratinases are extracellular proteases capable of catalyzing the degradation of insoluble keratin-rich substrates such as feathers, hair, and nails, which are highly stable structures due to their high content of disulfide bonds, making them resistant to most common proteases [4, 19, 37]. These enzymes, generally classified as serine or metalloproteases, have been investigated in ligninolytic fungi such as *Pleurotus* spp., whose enzymatic diversity reveals applications in sustainable processes [2, 4, 10].

The products of keratin hydrolysis are mainly amino acids and smaller peptides. These compounds can be applied in various sectors, such as in the production of fertilizers, in the pharmaceutical and food industries, in environmental pollution control, and in leather processing, among others [10,43].

For example, in *P. pulmonarius*, a 16 kDa protease with proven keratinolytic activity on feathers, hair, and human keratin, both *in vitro* and *in vivo*, was identified, indicating its value in the bioconversion of keratinous waste [4, 43]. Its effectiveness has also been demonstrated in lignocellulosic substrates, extending its scope beyond keratins [12]. In addition, enzymes from *P. ostreatus* and *P. sapidus* have been associated with the degradation of structural proteins in lignin-rich environments, which highlights the adaptive role of these proteases in various biotechnological systems [2, 11, 37].

The application of *Pleurotus* spp. proteases in protein hydrolysis has proven effective in increasing digestibility and generating bioactive peptides with functional potential. Alkaline proteases obtained from *P. eryngii* were applied in the pretreatment and promoted an increase of up to 7.54 times in the release of amino acids, with emphasis on the L isomers of lysine and arginine, in addition to the generation of up to 19,870 short peptides (<800 Da), which are highly bioavailable. This result is noteworthy because the amino acids mentioned are important in the context of human health and muscle recovery. Making their bioavailability more efficient contributes to their nutritional value [10].

Proteases from *Pleurotus* spp. are promising biocatalysts for industrial applications, given their specificity, efficiency, and biodegradability [4, 19]. They are used in detergents, food, leather, silk, cosmetics, wastewater treatment, and metal recovery, such as silver. Their stability in alkaline pH favors their use as additives for detergents [37].

In this context, subtilisin PPP1 from *P. pulmonarius* has proven effective in removing organic stains, while the thermostable protease SPPS, isolated from *P. sajor-caju*, performs well under industrial conditions [18, 66]. Serine proteases, PoSl, are widely researched and dominate the industrial enzyme market [67]. In addition, native strains such as *P. albidus*, from the Amazon, produce high levels of proteases (34.00 U/mL), highlighting the regional value of these species [21].

6. Conclusion and future prospects

Pleurotus genus of fungi stands out as an important producer of proteases, whose biochemical properties offer broad potential for applications in areas such as medicine, agriculture, the food industry, and biotechnology. In addition to their functional diversity, these enzymes exhibit promising nematicidal potential and can be obtained from agro-industrial residual substrates, providing environmental and economic benefits by adding value to materials that would otherwise be discarded.

Despite recent advances, significant gaps remain in the understanding of the metabolic and molecular processes involved in the production of these enzymes, making it essential to explore new biotechnological strategies to enhance their efficiency and stability. Modern approaches, such as the immobilization of cells or enzymes on innovative supports, including nanoparticles, mesoporous materials, hydrogels, and structures derived from agro-industrial residues, have shown promising results by increasing catalytic activity, resistance to adverse conditions, and the potential for reuse in industrial processes.

In parallel, genetic engineering techniques offer pathways for obtaining more robust variants with higher yield and specificity, including in recombinant hosts, thereby expanding prospects for large-scale production. The growing demand for proteases with high stability, specificity, and sustainability creates a favorable environment for the development of innovative solutions based on *Pleurotus spp.*

Pleurotus proteases exhibit promising characteristics, including stability under alkaline conditions and high catalytic activity in challenging environments. These attributes enhance their potential for industrial, agricultural, and biomedical applications, highlighting their biotechnological value and reinforcing their potential as sustainable and competitive alternatives to existing market solutions.

Thus, the development of enzyme formulations derived from *Pleurotus spp.* for biochemical pest control, industrial processes, and high-precision biomedical applications represents a strategic pathway for innovation. Continuous investment in scientific research and technological development is essential to establish *Pleurotus* proteases as sustainable, innovative alternatives with economic and social relevance, promoting solutions aligned with current trends in biotechnology.

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3 CAPÍTULO 2

ARTIGO 2:

Linking the protease activity to the nematicidal action of edible mushroom

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COLABORADORES:

Adriane Toledo da Silva[1*], Debora Castro de Souza[1], Stefany Amorim de Souza[1], Jhennifer Cristina de Souza Alves[1], Eustáquio Souza Dias[2], Liliana Aguilar-Marcelino[3] Filippe Elias de Freitas Soares[1]

1 Departamento de Química, Laboratório de Biotecnologia e Bioquímica Aplicada, Universidade Federal de Lavras

2 Departamento de Biologia, Universidade Federal de Lavras

3 Centro Nacional de Investigação Disciplinar em Saúde e Segurança Animal, Instituto Nacional de Investigação Florestal, Agrícola e Pecuária (INIFAP)

*Corresponding author – filippe.soares@ufla.br

ABSTRACT

Biological control using edible mushrooms as natural enemies is a sustainable alternative for pest management. Despite the well-established literature on toxins and secondary metabolites

produced by these fungi in the biochemical control of nematodes, the nematicidal activity of proteases from different *Pleurotus* species is yet to be investigated. Therefore, this study aimed to correlate protease to the nematicidal activity of different mushrooms, *Pleurotus* sp., *P. ostreatus* (SB), *P. ostreatus* (Pearl), and *P. djamor*. For such a purpose, we performed motility assays of *Panagrellus* sp. at different time intervals, 6, 12, and 24 hours for each of the mushrooms. In addition, the protease activity was measured using different pH (5, 7, and 9) and fermentation time intervals (45 and 75 days). Furthermore, we also evaluated the effect of this cell-free extract on *Panagrellus* sp. In response to these experiments, all edible mushrooms showed a reduction over 82% for the nematode-feeding activity ($p<0.01$). The cell-free crude extract of each of the fungi studied showed nematocidal activity ($p<0.01$). For the 45-day fermentation, *P. djamor* exhibited statistical significance ($p<0.01$) compared with the others, reaching a reduction percentage of 73%. For the 75-day fermentation, *Pleurotus* sp. and *P. ostreatus* (Pearl) showed significant differences compared with the other fungi ($p<0.01$), with reduction percentages of 64 and 62%, respectively. Herein, protease activity was associated with the nematicidal action of different *Pleurotus* species in controlling *Panagrellus* sp.

Keywords: enzymes, edible mushroom, biological control, nematophagous fungi

1. INTRODUCTION

Parasite nematodes are a major cause of crop loss worldwide, accounting for 10-14% of total losses due to the infections they cause in plants. In Brazil, it is estimated that the damage costs approximately US$ 6.5 billion a year (Ha et al., 2017; Machado et al., 2015; Soares et al., 2023). In this context, chemical control has been used to reduce crop losses (1.3 dichloropropene, fluensulfone, and fluazaindolizine) (Phani et al., 2021; Eugui et al., 2022). However, the indiscriminate use of these substances raises concerns about the presence of harmful residues in food, which is a potential threat to human health. In response to these challenges, new alternatives for nematode control have been developed to reduce the impact of agrochemicals (Sehrawat et al., 2022).

In this context, the use of biological agents has emerged as an alternative. Biological control is described as a strategy to control pests by using living organisms (Abdelaziz et al., 2023). Several types of fungi, such as basidiomycetes, are called nematode-trapping fungi based on their many strategies to prey on nematodes that are a source of nutrients, mainly nitrogen (Rodríguez-Barrera et al., 2021).

Nematophagous fungi have five types of strategies to control nematodes 1) predators, 2) ovicides, 3) endoparasites, 4) toxin producers, 5) producers of specialized attack devices) (Braga and Araújo, 2014; Soares et al., 2018). *Pleurotus* mushrooms species are toxin producers. Consequently, extracts from different *Pleurotus* species can be employed for the biochemical control of nematodes. Wille et al. (2019) have demonstrated that such extracts reduced the reproduction of the root-knot nematode *Meloydogyne* incognita in lettuce by 70%. Moreover, these fungi produced several enzymes (Al-Ani et al., 2022; Sufiate et al., 2017).

Proteases are important for catalyzing the digestion of nematodes since their cuticle is mostly composed of proteins (Braga and Araújo, 2014; Soares et al., 2018). To overcome this barrier, nematophagous fungi developed mechanical and enzymatic strategies. Protease (EC

3.4) is the main enzyme involved in this process. This macromolecule is responsible for catalyzing the hydrolysis of peptide bonds that are present in the cuticle protein (Soares et al., 2023).

In this context, this study aimed to link protease activity to the nematicidal activity of *Pleurotus* spp.

2. MATERIAL AND METHODS

2.1 Organisms

We obtained different isolates of the edible mushrooms used in all experiments, *Pleurotus* sp., *P. ostreatus* (SB), *P. ostreatus* (Pearl), and *P. djamor*, from the Fungal Culture Collection of the Laboratory of Edible Mushrooms, Department of Biology at the Federal University of Lavras (UFLA) (21º 14' 43" S Longitude: 44º 59' 59" W).

These fungi were maintained on Petri dishes containing PDA, under refrigeration, in the dark. For the solid-state fermentation of these fungi, wheat grains were used as a culture medium. A mass of 7 kg of these grains was soaked in water for 24 h. After soaking, the hydrated grains were mixed with 1 kg of sawdust, 500 g of wheat bran, and 500 g of limestone. The medium was then added to glass jars and covered with aluminum foil. The containers were then autoclaved for 1 h at 121 °C. This step was repeated after 24 h. After letting the jars cool to room temperature, the medium was inoculated with mycelium of each mushroom grown for 7 days on PDA plates (Siqueira et al., 2011; 2012). These containers were incubated for a period of 45 and 75 days at room temperature.

Nematodes of the genus *Panagrellus* were obtained commercially and maintained on Petri dishes containing oats and water in the Laboratory of Applied Biochemistry and Biotechnology at the Department of Chemistry at the Federal University of Lavras (UFLA) (Sufiate et al. 2017).

2.2 Nematophagous activity of edible mushrooms

A culture medium based on 2% agar water (w/v) was prepared. The medium was placed in Petri dishes and then each mushroom species was inoculated. The mushrooms were maintained in this culture medium for 10 days, at a temperature of 28 °C±1 and without the presence of light.

After this period, the nematode-trapping assay was performed (Sufiate et al. 2017) using *Panagrellus* sp. model nematodes. Subsequently, a solution of these nematodes was prepared.

Six experimental groups were formed: five treated groups and one control group. Each treated group contained different mushroom isolates, *Pleurotus* sp., *P. ostreatus* (SB), *P. ostreatus* (Pearl), and *P. djamor*, in addition to approximately 100 juvenile *Panagrellus* sp. The control group consisted only of the plate containing agar water and around 100 juvenile *Panagrellus* sp., without fungus.

These plates were maintained at a temperature of 28 °C±1, in the dark, at different incubation times (6 h, 12 h, and 24 h). Six replicates were produced per mushroom species. After the time intervals of 6 h, 12 h, and 24 h, the nematodes were counted using an optical microscope with a 10x objective. The count was based on the number of intact juveniles in 10 random fields in each plate, as described by Sufiate et al. (2017).

2.3 Assays with extracts of mushrooms rich in enzymes

2.3.1. Enzyme extraction

To extract the enzymes and obtain the cell-free extract, distilled water was added to the solid media described in section 2.1, obtained after the growth of the fungi in two different incubation periods, 45 days and 75 days, in a 1:5 (m/v) ratio. The media were then stirred at 180 rpm at 25°C±1 for 1 h. Subsequently, they were filtered using filter paper and centrifuged

at 10,000 *g* for 12 min. Then, the cell-free supernatant was collected and the protease enzymatic activity and nematocidal activity were evaluated (Soares et al. 2010).

2.3.2. Enzyme activity

Enzyme activity was measured using the caseinolytic assay (Braga et al., 2011). For this assay, a 0.5 mL solution of 1% (m/v) casein, 0.4 mL of buffer, and 0.1 mL of previously prepared extract were incubated at 50 °C for 60 min in test tubes. This assay was used to determine protease activity produced by *Pleurotus* sp., *P. ostreatus* (SB), *P. ostreatus* (Pearl), and *P. djamor*. Protease activity was measured at different pH using the following buffers: 50 mM citrate (pH 5) and 50 mM Tris-HCl (pH 7 and 9).

After 60 min, the reaction was stopped by adding 1 mL of 10% (m/v) trichloroacetic acid (TCA). Time zero (T0) was also prepared by using the same above-mentioned conditions, but adding TCA first. Immediately afterward, the solutions were centrifuged at 10,000 *g* for 12 min. The supernatant was recovered for reading in a UV-Vis spectrophotometer at 280 nm. One enzyme unit was defined as the amount of protease needed to release 1 µg of tyrosine, per minute, under assay conditions.

2.3.3. Nematicidal activity

An assay was performed to evaluate the nematicidal activity of the crude extract of the edible mushrooms *Pleurotus* sp., *P. ostreatus* (SB), *P. ostreatus* (Pearl), and *P. djamor*. For this assay, *Panagrellus* genus model nematodes were used (Ferreira et al. 2019).

Ten experimental groups were formed: nine treated groups, four groups using extracts with the enzymes denatured (previously boiled for 2 h), four groups using extracts with the active enzymes, and one control, without extract, containing only distilled water. Each treated group contained 50 µL of the extracts, in addition to approximately 50 juvenile *Panagrellus* sp. The control group contained only 50 µL 50 µL of water and around 50 juvenile *Panagrellus* sp.

The microtubes were kept for 48 h at 28 °C±1 in the dark. Then, the number of intact juveniles contained in each microtube was counted on an optical microscope with a 10x objective. The nematode was considered intact if its cuticle showed neither visible damage nor extravasation of the internal contents.

The experiment was performed using two different extracts, obtained at different fermentation times (45 and 75 days) for each of the fungal isolates.

2.4. Statistical analysis

Data statistical interpretation was performed using analysis of variance within the significance levels of 1 and 5% of probability, also in addition to Tukey's test at levels of 1 and 5% of probability on the BioEstat 5.3 Software (Ayres et al., 2003).

In addition, the average percentage reduction in the number of *Panagrellus* sp. juveniles was calculated using the following equation (Mendes et al., 2023):

$$\%\text{Reduction} = \frac{\overline{\text{x}}_{Panagrellus\ \text{control}} - \overline{\text{x}}_{Panagrellus\ \text{treatment}}}{\overline{\text{x}}_{\text{P}anagrellus\ \text{control}}}$$

3. RESULTS

3.1 Nematophagous activity of edible mushrooms

Fig. 1 shows the nematophagous efficacy of edible mushrooms against *Panagrellus* sp. at different time intervals (6 h, 12 h, and 24 h). Table 1 indicates the respective percentages of reduction.

The analysis of the nematophagous activities of different mushrooms at 6 h showed statistical significance. In contrast, by comparing each mushroom separately with the control group, *Pleurotus* sp. and *P. ostreatus* (SB) showed a significant reduction ($p<0.01$) regarding their respective controls. *P. djamor* and *P. ostreatus* (Pearl) showed no statistical significance compared with their respective control groups ($p>0.05$) (Fig. 1A). The percentages of reduction

for *Pleurotus* sp., *P. ostreatus* (SB), *P. ostreatus* (Pearl), and *P. djamor* were 67±11, 44±17, 0, and 35±23%, respectively (Table 1).

At 12 h, *P. ostreatus* (SB) and *P. djamor* showed significant differences ($p<0.01$) compared with the other edible mushrooms in terms of nematophagous activity. By comparing each treated group separately with the control group, all groups showed statistical significance ($p<0.01$) (Fig. 1B), with reduction percentages of 75±7, 94±2, 80±2, and 88±6%, respectively, for *Pleurotus* sp., *P. ostreatus* (SB), *P. ostreatus* (Pearl), and *P. djamor* (Table 1).

Finally, at 24 h, none of the treated groups showed statistical significance ($p>0.05$) compared with the other groups. By comparing each treated group separately with the control group, all groups showed statistical significance ($p<0.01$) (Fig. 1C), with reduction percentages of 95±3, 95±4, 94±1, and 82±9%, respectively, for *Pleurotus* sp., *P. ostreatus* (SB), *P. ostreatus* (Pearl), and *P. djamor* (Table 1).

Fig. 2 shows the nematophagous action of the fungi. These images illustrates the degradation of the nematode's cuticle and the presence of possible toxin droplets produced by the fungi.

3.2 Experiments with extracts from enzyme-rich edible mushrooms

The enzymatic activity of the cell-free extracts of the fungi was measured at different pHs (5, 7, and 9). In addition, it is worth noting that we also evaluated the protease activities of extracts from the solid-state fermentation of the mushrooms at different times (45 and 75 days).

Table 2 shows the enzymatic activity in U mL^{-1} for the mushrooms *Pleurotus* sp., *P. ostreatus* (SB), *P. ostreatus* (Pearl), and *P. djamor* for 45 days and 75 days.

For the time interval of 45 days at pH 5, all crude extracts produced by the fungi showed proteolytic activity. The proteolytic activity of *Pleurotus* sp. increased from 3.5 U mL^{-1} to 15 U mL^{-1} by increasing the fermentation time from 45 days to 75 days. *P. ostreatus* (SB) showed

the same profile, with an increase in proteolytic activity from 3.6 to 5.0 U mL^{-1}. *P. ostreatus* (Pearl) was showed the same profile, with an increase in proteolytic activity from 2.8 U mL^{-1} (45 days) to 4 U mL^{-1} (75 days). However, *P. djamor* had a proteolytic activity of 8.3 U mL^{-1} for the 45-day fermentation but no proteolytic activity for the 75-day fermentation. Only *Pleurotus* sp. showed proteolytic activity at pH 7. However, for the 45-day fermentation, its proteolytic activity reached 6 U mL^{-1}, increasing by 10 U mL^{-1} at 75 days of fermentation. None of the edible mushrooms showed proteolytic activity at pH 9 (Table 2).

No significant difference ($p>0,05$) was found by comparing all proteolytic activity values obtained for *P. ostreatus* (SB) and *P. ostreatus* (Pearl) at pH 5 at the different fermentation times. For *P. djamor,* the 45-day fermentation at pH 5 was the optimal condition for enzyme production since the fungus produces no enzymes at the other time intervals and pH analyzed (Table 2).

The edible mushroom *P. djamor* ($p<0.01$) showed the highest production of proteolytic enzymes for the 45-day fermentation. In turn, for the 75-day fermentation, *Pleurotus* sp. ($p<0.01$) showed the highest proteolytic activity (Table 2).

Fig. 3 shows the nematicidal efficiency of each active crude extract from *Pleurotus* sp., *P. ostreatus* (SB), *P. ostreatus* (Pearl), and *P. djamor* against *Panagrellus* sp. at different fermentation times in solid state (45 and 75 days).

According to the evaluation of the nematocidal efficiency of the different active crude extracts from the edible mushrooms for the 45-day solid-state fermentation, the extract produced by *P. djamor* showed a significant difference compared with the others ($p<0.01$). By comparing each mushroom separately with the control group and the denatured crude extract, *Pleurotus* sp. ($p<0.05$), *P. ostreatus* (Pearl), and *P. djamor* ($p<0.01$) showed a significant reduction. *P. ostreatus* (SB) showed no statistical significance compared with the control group

($p>0.05$); however, compared with the denatured group, a statistical significance was found ($p<0.05$) (Fig. 3A).

For 75-day solid-state fermentation, *Pleurotus* sp. and *P. ostreatus* (Pearl) showed a significant difference compared with the others ($p<0.01$). In turn, the comparison of each mushroom species separately with the control group and the denatured crude extract showed a significant reduction ($p<0.01$) in *Pleurotus* sp. and *P. ostreatus* (Pearl). *P. djamor* and *P. ostreatus* (SB) showed no statistical significance compared with the control group and the denatured group ($p>0.05$) (Fig. 3B).

Based on such promising results and the production of toxins, mushroom species also produce enzymes with nematocidal effects. Fig. 4 illustrates an example of the digestion of *Panagrellus* sp. due to the presence of enzymes in the crude cell-free extract of *P. ostreatus* (Pearl) (Fig. 4A) and *P. djamor* (Fig. 4B).

4. DISCUSSION

The production of toxins is the main mechanism of action for mushrooms of the genus *Pleurotus* (Hibbett and Thorn et al., 1994). The droplets are rich in substances derived from linoleic acid, with a toxic effect on nematodes. These toxins can paralyze or even kill the host (Soares et al., 2018), which corroborates our results, according to Fig. 2, since all the mushrooms evaluated produced toxins against *Panagrellus sp*.

In addition to toxins, mushrooms also produce proteases, which are extracellular hydrolytic enzymes produced by nematophagous fungi (Ferreira et al., 2019). These fungi play a significant role as antagonistic microorganisms, thus representing a valuable option for the biological control of larvae in various environments. The infection process occurs through the action of hydrolytic enzymes, a strategy whose effectiveness is strongly associated with proteases (Bonants et al., 1995; López-Llorca et al., 1993).

The proteases produced by fungi can show optimum activity at different pHs depending on their acidic, neutral and basic classification. Acid proteases have maximum activity in the pH range between 2.0 and 6.0, neutral proteases between 6.0 and 8.0, and basic proteases between 8.0 and 13 (Liu et al., 2023; Soares et al., 2023).

Genier et al. (2015) reported the maximum activity of the serine protease enzyme from *P. ostreatus* occurring at pH 9. Our study found the proteolytic activity of the mushrooms occurring at pH 5 and 7. Therefore, isolates of the same species have different effects in terms of their enzymatic and nematocidal activity. According to Mendoza-de-Gives (1999), each fungal isolate can have a different action profile, thus showing the importance of evaluating enzymatic production for several isolates.

The nematocidal assay (Table 1) indicated that by linking the nematocidal activity of *Pleurotus* sp. and *P. ostreatus* (Pearl) to enzymatic activity, the reduction percentage of *Panagrellus* juveniles reached 95% and 95%, respectively, at 24 h. In turn, the assay using the cell-free extract found the highest reduction percentages of 64% and 62%, respectively (Table 3). Such a difference in values suggests that *P. ostreatus* has at least two action mechanisms against nematodes. The toxins were important for paralyzing the nematodes, whereas the enzymes were important for the digesting process the cuticle. Our results suggest that the enzymatic action is an important mechanism in the infection process for this fungus, since over 60% of the nematode reduction was caused by the enzymes (Genier et al., 2015).

In the case of *P. ostreatus* (SB), the action mechanism occurs differently from the isolates *Pleurotus* sp. and *P. ostreatus* (Pearl). At 24 h, the percentage reduction of *Panagrellus* sp. reached 95% in the nematocidal assay (Table 1). In the enzymatic assay, the highest reduction was 19% (Table 3). Such a results suggests that the main action mechanism of this fungus is the production of toxins, as reported by Yang et al. (2007). These values show that the nematocidal activity may vary between strains even in the same species.

In this context, Wille et al. (2019) reported that the aqueous extract of *P. ostreatus* showed a 100% reduction in second-stage juveniles of *Meloidogyne incognita*, after 12 days of in vitro incubation. Herein, the extract produced by this same species of edible mushroom achieved a 60% reduction in *Panagrellus* sp. However, the maximum incubation time evaluated was reached at 24 hours, suggesting that a longer incubation time favors a higher reduction percentage.

The reduction percentages for *Panagrellus* sp., 95, 94, and 95% for the mushrooms *Pleurotus* sp., *P. ostreatus* (Pearl), and *P. ostreatus* (SB) (Table 1) at 24 h corroborate the results found by Genier et al. (2015). For the same time interval, the authors found a reduction percentage of 95% for *Panagrellus* sp. in the nematophagous assay. In turn, the enzymatic assay of *Pleurotus* sp. and *P. ostreatus* (Pearl) showed identical results. Our study found a percentage reduction of 64% and 62%, respectively, for the same mushrooms, while Genier et al. (2015) reported a 65.6% reduction in nematodes (Table 3).

Furthermore, the nematocidal effect of *P. eryngii* and its crude extract were evaluated on juveniles of *Panagrellus* sp. in the 24-h in vitro incubation period (Sufiate et al. 2017). The following reduction percentages were obtained, respectively: 60 and 90%. These results are close to those found in the present study, for the different species of *Pleurotus* used (Tables 1 and 4).

P. djamor stands out in the production of proteases among the edible mushrooms studied. Therefore, the its activity and nematocidal action measurement suggest that its nematocidal action is linked to its enzyme production. The nematophagous assay showed a reduction percentage of 88% for *Panagrellus* sp. only for the time interval of 12 h (Table 1). In turn, the enzymatic assay found a reduction percentage of 73% for *Panagrellus* sp. (Table 3).

Research has used *P. djamor* promisingly for biological control of the nematode. Pineda-Alegría et al. (2017) used the hydroalcoholic crude extract of *P. djamor* to control

infective larvae and eggs of *Haemonchus contortus*. The authors found the following secondary metabolites produced by these fungi: fatty acids pentadecanoic, hexadecanoic octadecadienoic, octadecanoic acid, and terpene β-sitosterol. These metabolites were responsible for the 98.7%reduction of *H. contortus* larvae.

Lopes et al. (2023) used spent mushroom substrates (SMS) from *P. djamor* still containing secondary metabolites to control *Meloidoyne javanica*. According to the authors, the lowest concentration of the residues (15% w/w) was responsible for a 100% reduction in the presence of nematodes in the roots, as well as the reproduction factor of these pests. The study did not evaluate the nematophagous action mechanism of these fungi. However, according to our study, the control of these pests may have occurred due to the presence of toxins and the proteases produced by the fungus.

The literature have no reports on the enzymatic action of *P. djamor* since the above-mentioned studies discussed only the role of toxins in biological control. Our assays revealed that, in addition to toxins, proteases also played an important role in controlling nematodes for this fungus.

The results obtained for *Pleurotus* sp., *P. ostreatus* (SB), *P. ostreatus* (Pearl), and *P. djamor* show their nematicidal activity against juveniles *Panagrellus* sp. Amid these promising results, the use of those fungi is interesting for new studies involving other species of nematodes that parasitize plants and animals, as well as other pests.

TABLE

Table 1 Percentage reduction in *Panagrellus* sp. in water agar (2% WA) in the groups treated with the different mushrooms species, *Pleurotus* sp., *P. ostreatus* (SB), *P. ostreatus* (Pearl), and *P. djamor*, during the intervals of 6, 12, and 24 hours, compared with the control group without fungi.

[a,a] Identical letters in the same column indicate no significant difference ($p>0.05$) between the time intervals.a,b

Reduction of nematodes (%)				
Interval (hours)	*Pleurotus* sp	*P. ostreatus* (SB)	*P. ostreatus* (Pearl)	*P. djamor*
6	67±11a	44±17a	0a	35±23a
12	75±7a	94±2b	80±2b	88±6b
24	95±3b	95±4b	94±1b	82±9b

Different letters in the same column indicate a statistical difference between the time intervals ($p<0.05$).

Table 2 Enzymatic activities (expressed as U mL^{-1} of extract) of the mushrooms, *Pleurotus* sp., *P. ostreatus* (SB), *P. ostreatus* (Pearl), and *P. djamor*, at the pHs evaluated (5, 7, and 9) after 45 and 75 days of solid-state fermentation.

	Enzymatic activity (U mL^{-1})							
	Pleurotus sp.		*P. ostreatus* (SB)		*P. ostreatus* (Pearl)		*P. djamor*	
pH / Interval (days)	45	75	45	75	45	75	45	75
5	3.5 ± 0,3a*	15 ± 1b*	3.6 ± 0.2a	5.0 ± 0.9a	2.8 ± 0.4a	4 ± 2a	8.3 ± 0.2	-
7	6 ± 1**	10 ± 1**	-		-		-	-
9	-		-		-		-	-

*,** Different numbers of asterisks for the same mushroom and the same incubation time indicate a significant difference between the enzyme activity values for the different pH ($p<0.01$). [a,b] Different letters for the same mushroom and pH indicate a significant difference between the incubation times evaluated ($p<0.01$).

Table 3 Percentage reduction of *Panagrellus* sp. in the groups treated with the different crude extracts of the fungi, *Pleurotus* sp., *P. ostreatus* (SB), *P. ostreatus* (Pearl), and *P. djamor*, compared to the control group without fungi, after fermentation in the solid state for 45 and 75 days.

Reduction of nematodes (%)				
Interval (days)	*Pleurotus* sp	*P. ostreatus* (SB)	*P. ostreatus* (Pearl)	*P. djamor*
45	27±13a	19±15a	28±10a	73±6a
75	64±16b	15±3a	62±23b	8±6b

[a,a] Identical letters in the same column indicate no significant difference ($p>0.05$) between the time intervals.[a,b] Different letters in the same column indicate a statistical difference between the time intervals ($p<0.05$).

FIGURE

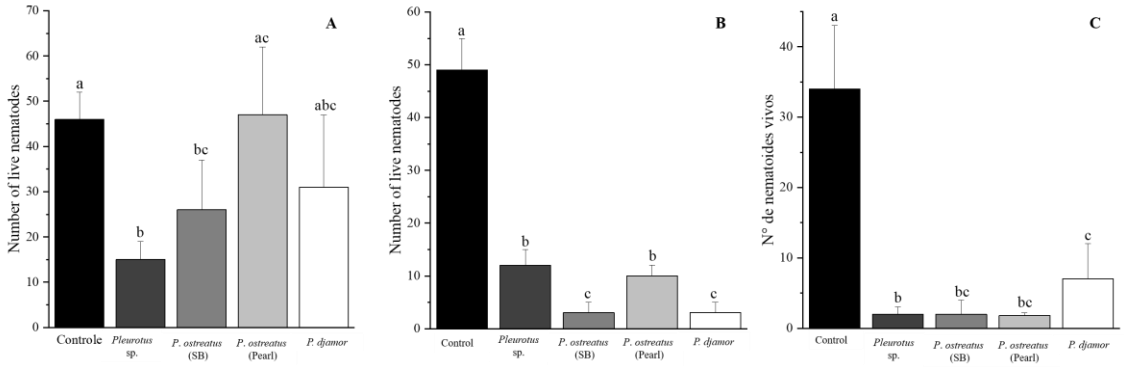

Fig. 1 Evaluation of nematophagous activity of *Pleurotus* sp., *P. ostreatus* (SB), *P. ostreatus* (Pearl), and *P. djamor* mushrooms against *Panagrellus* sp. at 6, 12, and 24 hours (a, b, and c). Identical letters indicate no significant difference between the groups evaluated ($p>0.05$). Different letters indicate a significant difference between the groups evaluated ($p<0.05$).

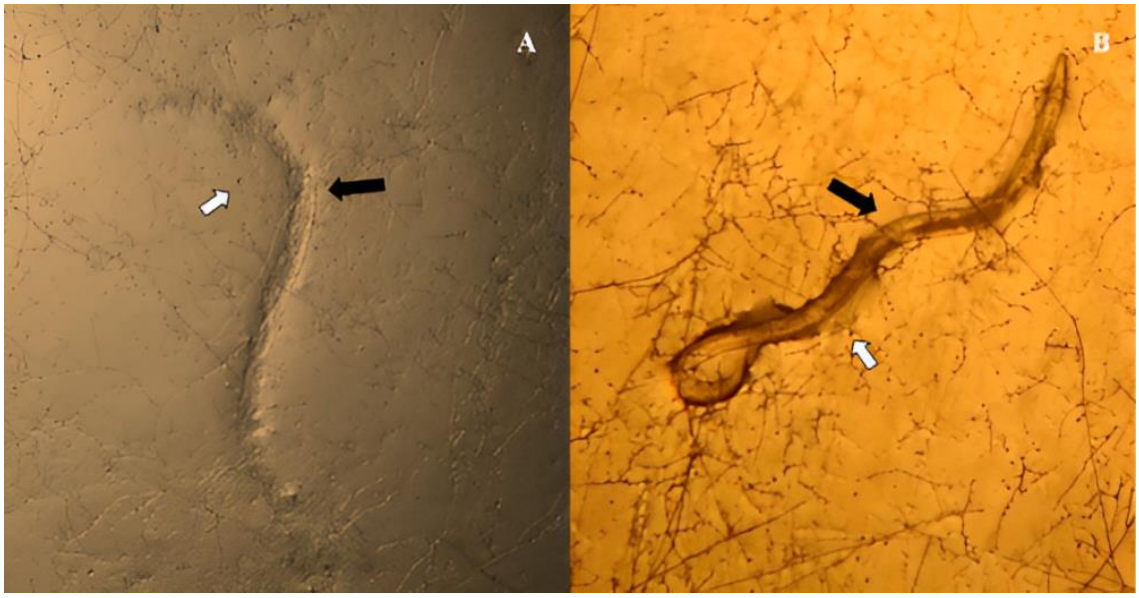

Fig. 2 Nematicidal activity of mushrooms (a) *Pleurotus* sp., (b) *P. djamor*. Black arrows: degraded cuticle of the nematodes. White arrows: possible droplets of toxins produced by the mushrooms to paralyze the nematode.

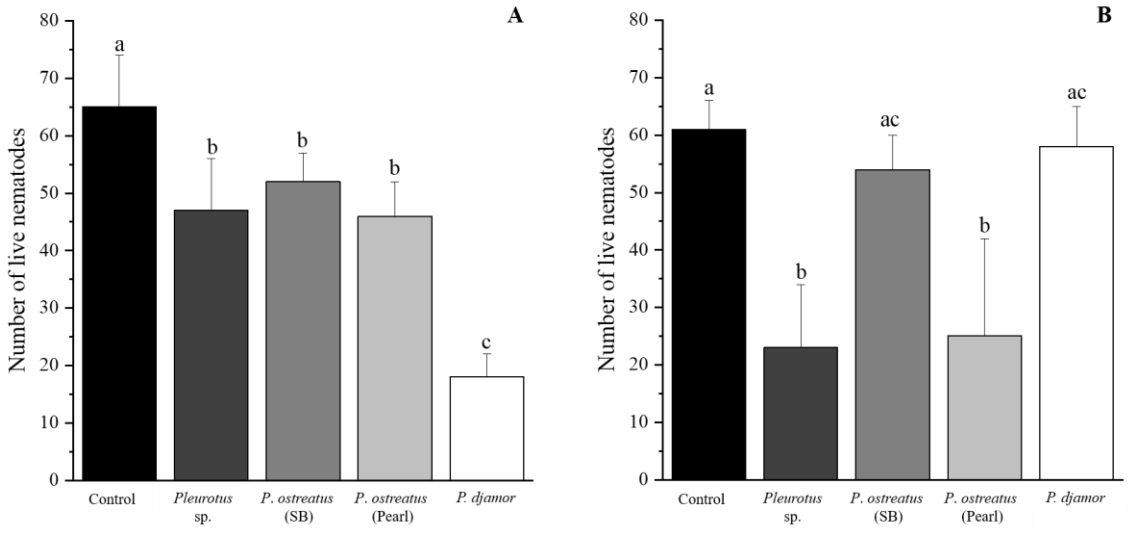

Fig. 3 Evaluation of the nematicidal activity of the active crude extracts of the mushrooms against *Panagrellus* sp. after 45 days (a) and 75 days (b). Identical letters indicate no significant difference between the groups evaluated ($p>0.05$). Different letters indicate a significant difference between the groups evaluated ($p<0.05$).

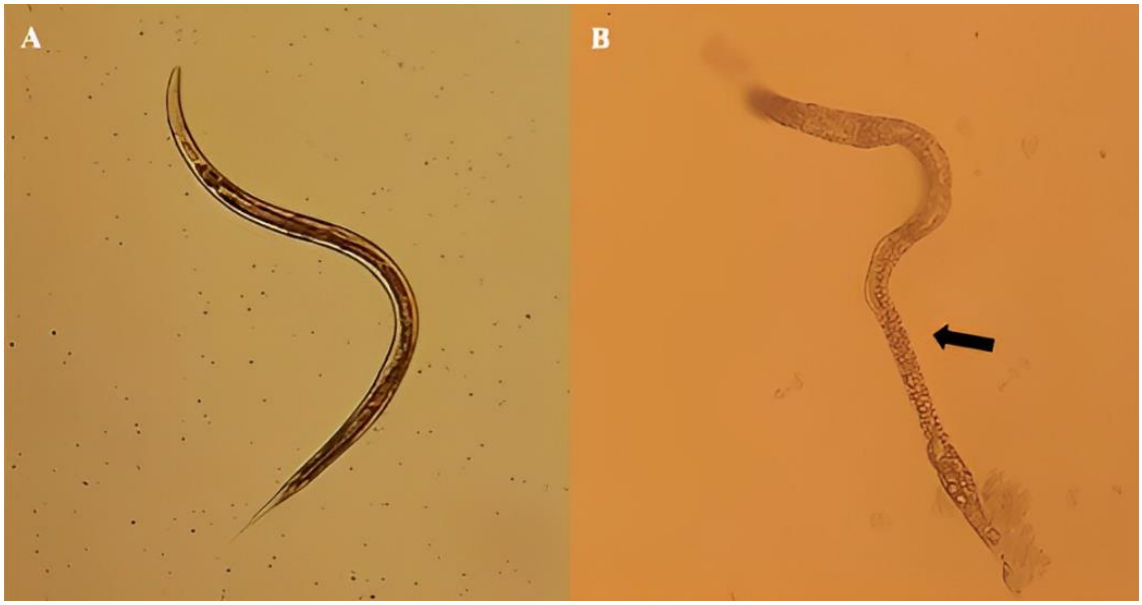

Fig. 4 Ilustration of the nematicidal activity of the active crude extract of the mushrooms. A: Control group and B: Treated group

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4 CAPÍTULO 3

ARTIGO 3:

Evaluation of the nematicidal and enzymatic activity of *Pleurotus djamor* on *Trichostrongylus* spp. and *Strongyloides papillosus*

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COLABORADORES:

Adriane Toledo da Silva[1*], Lisseth Bibiana Puentes Figueroa[1], Debora Castro de Souza[1], Eustáquio Souza Dias[2], Laisa Bastos Albuquerque[3], Tiago Facury Moreira[3], Fabio Ribeiro Braga[4], Filippe Elias de Freitas Soares[1]

1 Departamento de Química, Laboratório de Bioquímica e Biotecnologia Aplicada, Universidade Federal de Lavras

2 Departamento de Biologia, Universidade Federal de Lavras

3 Escola de Veterinária, Departamento de Clínica e Cirurgia Veterinária, Universidade Federal de Minas Gerais

4 Laboratório de Parasitologia Experimental e Controle Biológico, Universidade Vila Velha

*Corresponding author – filippe.soares@ufla.br

ABSTRACT

Parasitic gastrointestinal nematodes (GINs) cause diseases that are a major challenge to livestock production. Chemical control is the most widely used method, but it has many economic and environmental problems. This study was to evaluate *in vitro* the nematicidal and enzymatic activity of *Pleurotus djamor* against *Trichostrongylus* spp., and *Strongyloides papillosus.* The fungus was cultivated in solid medium composed of wheat seeds for 25 days. The enzyme extraction was then performed using distilled water in the ratio of 1:5. Subsequently, by means of filtration and then centrifugation, the cell-free crude extract (CFCE) was obtained. Proteolytic activity was measured at pH 5 using the caseinolytic assay. The nematophagous assay of the *P. djamor* on the infective larvae (L_3) was performed for 6 h. The nematicidal effect of the CFCE (active and denatured) was analyzed over a period of 48 hours. The CFCE showed proteolytic activity of 8.9 ± 0,5 U mL^{-1}. *P. djamor* reduced the number of L_3 by 75% ($p<0.01$). The active CFCE showed statistical difference compared to the control and the denatured ($p<0.01$). On the other hand, there was no significant difference between the denatured group and the control group (without enzymes). The percentage reduction of L*3* was 57%. This result suggests that the nematicidal action occurred mainly due to the activity of the proteases present in the CFCE. This study reports for the first time the applicability of proteases produced by *P. djamor* in the biochemical control of GINs, which could become an alternative biopesticide in the future.

Keywords: protease, fungus, nematicidal, parasite.

1. INTRODUCTION

According to Braga et al. (2020) and Vilela et al. (2021), gastrointestinal parasitic nematodes are one of the main obstacles to ruminant farming in Brazil and worldwide. As a direct consequence, in addition to compromising the health of ruminant herds, they cause high

costs with anthelmintic treatments, which is most of the time uncertain and provides parasitic resistance (Grisi et al., 2014; Kaplan et al., 2020; Tachack et al., 2022).

In view of this impasse, it becomes essential to study and apply environmentally sustainable and effective alternatives in the control of gastrointestinal parasitic nematodes. Among these alternatives, biological control using "natural enemies" such as nematophagous fungi has been a promising option (Fonseca et al. 2023). In addition, many of these fungi are capable of affecting nematodes by different mechanisms, such as through the formation of traps, production of toxins, and enzymes (Braga et al., 2011; Sivanandhan et al., 2017; Soares et al., 2019; Aguilar-Marcelino et al., 2023).

In vitro use of *Pleurotus* spp. as biocontrol of parasitic nematodes has been reported in the literature. Its activity is concentrated in the production of toxins (Aguilar-Marcelino et al., 2017; Rodriguez-Barrera et al., 2021). The species *Pleurotus djamor* is an edible mushroom with reports in the literature of its toxin production in the control of gastrointestinal nematodes (GIN) (Pineda-Alegria et al., 2017; Colmenares-Cruz et al., 2021; González-Cortázar et al., 2021). However, there are still no reports of the use of enzymes produced by this fungus for the *in vitro* control of *Trichostrongylus* spp., and *Strongyloides papillosus*. Protease (EC 3.4) is the main enzyme responsible for the process of degradation of the cuticle of these parasites (Soares et al., 2023).

Thus, the present study aimed to investigate the nematophagous and proteolytic activity of *P. djamor* on *Trichostrongylus* spp. and *S. papillosus.*

2. MATERIALS AND METHODS

2.1 Organisms

The edible mushroom *P. djamor* isolate used in all experiments was obtained from the Fungal Culture Collection of the Laboratory of Edible Mushrooms, Department of Biology at the Federal University of Lavras (UFLA).

Solid-state fermentation of this species was carried out using wheat seeds as a culture medium (Siqueira et al., 2011; 2012). After preparing the medium, the flasks were incubated for 25 days, under refrigeration, in the dark. The maintenance of these fungi was carried out in Petri dishes containing 2% potato-dextrose-agar (2% PDA) (w/v). These plates were also kept refrigerated, in the dark.

Infective larvae (L_3) of *Trichostrongylus* spp. (62%) and *S. papillosus* (38%) were obtained from naturally infected goat faeces from the School of Veterinary Medi cine of the Federal University of Minas Gerais, Brazil (UFMG).

2.2 Nematophagous assay

Solid culture medium 2% water-agar (2%WA) (w/v) was used. This medium was poured into Petri dishes and after solidifying, *P. djamor* was inoculated. These plates were incubated at 28 °C ± 1, for 10 days, in the absence of light.

After the growth of the fungi, a solution containing about 100 L_3 was added to the center of each plate. The assay consisted of two experimental groups, the treatment group and the control group, with 6 replicates for each group. The control group was formed by the agar-water plate and the larvae solution, without the presence of the fungus. The plates were kept in the dark, at 28 °C, for 6 hours (Silva et al., 2024). Immediately afterwards, the count of intact L_3 in ten random fields per plate was performed by optical microscopy, at 10x objective (Sufiate et al., 2017).

2.3 Enzymatic assays

2.3.1 Extraction

The extraction of enzymes in the solid medium described above (topic 2.1) was done using distilled water in the proportion of 1:5 (m/v). The suspension was shaken at 180 rpm for 1 hour at 25 °C ± 1. Then, a filtration was performed using Whatman No. 4 filter paper. Finally, to obtain the cell-free crude extract (CFCE), the filtrate was centrifuged for 12 minutes at 10,000 g. The supernatant was named CFCE. After obtaining the extract, enzyme and nematicidal assays were performed (Soares et al. 2010).

2.3.2 Enzymatic activity

The caseinolytic assay was used to measure the protease activity in the CFCE.In this assay, the conditions established were: temperature: 100 µL de CFCE, 50 °C, time: 60 min and pH 5. One unit of protease was defined as the amount of enzyme needed to release 1 µg of tyrosine per minute (U). To calculate the tyrosine concentration of CFCE, a calibration curve was constructed using an external standard with a concentration range of 20 to 160 µg mL^{-1} (Soares et al. 2019).

2.4 Nematicidal assay

To evaluate the nematicidal activity of the CFCE of *P. djamor*, three experimental groups were established: a control group (L_3 + distilled water) and two treatment groups: (L_3 + CFCE previously boiled for 2 hours, with denatured enzymes) and (CFCE with active enzymes + L_3). In each plate, about 60 L_3 were added and for each group, 10 replicates were made. After 48 hours, the intact L_3 were counted with the aid of an optical microscope at 10x objective (Sufiate et al., 2017). All experiments were repeated three times and all data were considered for statistical analyses.

2.5 Statistical analyses

In the nematophagous assay, there was only one pair of (independent) treatment samples to compare, so the data were statistically analyzed using one-way analysis of variance (ANOVA) at the 1% and 5% significance levels. In the nematicidal assay, there were at least 3 treatments, so data were statistically analyzed using one-way ANOVA and Tukey's test, both at the 1% and 5% significance levels (Ayres et al., 2003). The percentage reduction was calculated according to the method of Mendoza-de Gives et al. (1994).

3. RESULTS AND DISCUSSION

Figure 1 shows the nematophagous activity of *P. djamor* at 6 hours. According to the data obtained, the treatment group showed a significant difference in the number of L_3 compared to the control group ($p<0.01$). The percentage reduction of L_3 was 75%.

In Figure 2, the immobilization of nematodes by the fungus is noticeable. The hyphae are responsible for colonizing and destroying the L_3 (Rodriguez-Barrera et al., 2021). The presence of possible microdroplets of toxins produced by the fungus can also be observed, which are responsible for the nematophagous mechanism (Hibbertt and Thorn, 1994; Heydari et al., 2006; Silva et al., 2024). According to Hibbett and Thorn (1994), fungi of the genus *Pleurotus* produce toxins derived from linoleic acid.

The anti-helminthic activity of different fungi, *P. eryngii* (strains 1290 and 1291), *P. ostreatus* (strains 1123 and 0152), *P. cornucopiae* (strains 1328 and 1330), *Coprinus comatus* (strain 1103), *Panus sp.* (strain 801), *Lentinula edodes* (strain 401) and *L. boryanus* (strain 402), was evaluated against *Haemonchus contortus* larvae. The best results obtained for the larval mortality rate were: 88% (strain 0152), 88.5% (strain 1238), 91% (strain 1328), 93% (strain 1292) and 93% (strain 401). According to the authors, the nematophagous effect of the fungi may have been caused by the toxins they produce (Comans-Pérez et al., 2021). Some of these

toxins, such as acid trans-2-decenedioic, *p*-anisyl alcohol, 1-(4- methoxyphenyl)-1,2-propanediol, and 2-hydroxy (4'- methoxy)-propiophenone have been characterized and linked to the nematicidal activity of *Pleurotus* spp. (Kwok et al., 1992; Degenkolb and Vilcinskas, 2016).

In the work of Gonzaléz-Cortázar et al (2021), the presence of alitol and a terpene was identified in the hydroalcoholic extract of *P. djamor* fruiting bodies. These compounds were associated with nematicidal activity in the control of *H. contortus*, obtaining 92.56% reduction of larvae. In addition to these nematicidal metabolites, fatty acids also present in the crude hydroalcoholic extract of *P. djamor* showed an effect on *H. contortus* larvae. The following fatty acids were identified: pentadecanoic, hexadecanoic, octadecadienoic and octadecanoic acid, and a terpene, β-sitosterol. This evaluated extract was able to reduce 98.7% of larvae (Pineda-Alegria et al., 2017).

Nematode reduction results found by Comans-Pérez et al (2021) are superior to those in the present study; however, the incubation time was 5 days while in the present study it was only 6 hours. Therefore, the results obtained in the present study are promising, since a short time was enough for *P. djamor* to consume 75% of the larvae.

In the context of these positive results already described, it is noteworthy that the use of fungi in the control of gastrointestinal parasitic nematodes is an interesting alternative (Comans-Pérez et al., 2021). These studies relate the nematicidal activity of *P. djamor* to the production of toxins (Pineda-Alegria et al., 2017). However, in the present study, it is suggested that the nematicidal activity of *P. djamor* is not only related to the presence of toxins. In addition to toxins, fungi produce enzymes that participate in the degradation of the cuticle of these nematodes (Rodriguez-Barrera et al., 2021; Aguilar-Marcelino et al., 2023).

Enzymatic activity of the *P. djamor* was described by Sufiate et al. (2017), where a proteolytic activity of 32.74 U mL^{-1} was obtained. In another study, Genier et al. (2015) using

the same fungal genus reached more than 90 U mL^{-1} of proteolytic activity. In the present study, the crude cell-free extract (CCFE) of *P. djamor* had a proteolytic activity of 8.9 ± 0.5 U mL^{-1}.

In Figure 3, the important role of proteases in the infection process of gastrointestinal parasitic nematodes can be observed. The proteases present in the active CFCE were responsible for catalyzing the hydrolysis of peptide bonds in the proteins present in the cuticle of gastrointestinal parasitic nematodes (Sebastian et al., 2018; Soares et al., 2023). The denatured CFCE did not show a significant difference ($p>0.05$) from the control group. However, the active CFCE showed statistically significant difference ($p<0.01$) in relation to the control group and the denatured group. Thus, it is strongly suggested that the nematicidal action of the CFCE occurred due to enzymatic action. As protease activity was detected and measured in the CFCE, it is likely that these enzymes were responsible for the destruction and death of the nematodes. The percentage reduction of larvae compared to the control group was 57%. In Figure 4, immobilized nematodes and possibly the beginning of digestion of their cuticle can be observed.

The nematicidal activity on gastrointestinal parasitic nematodes linked to the presence of enzymes has already been reported by Soares et al. (2019). In that study, the percentage reduction of gastrointestinal parasitic nematodes (*Haemonchus* spp., *Cooperia* spp., and *Oesophagostomum* spp.) was 26%. This result and the present study demonstrate the significant nematicidal effect of proteases in the CFCE on L_3 of gastrointestinal parasitic nematodes. In this context, the CFCE of *P. djamor* is a promising alternative in the control of these parasites due to the presence of proteases.

4. CONCLUSION

Nematophagous activity of *P. djamor* by means of the production of toxins has already been described in the literature. However, the nematicidal action through *P. djamor* proteases

on *Trichostrongylus* spp. and *S. papillosus* was demonstrated in the present study for the first time, to the best of our knowledge. Thus, the results demonstrate the nematicidal potential of CFCE from this fungus, which could become, in the future, a new alternative for use as a biopesticide.

FIGURE

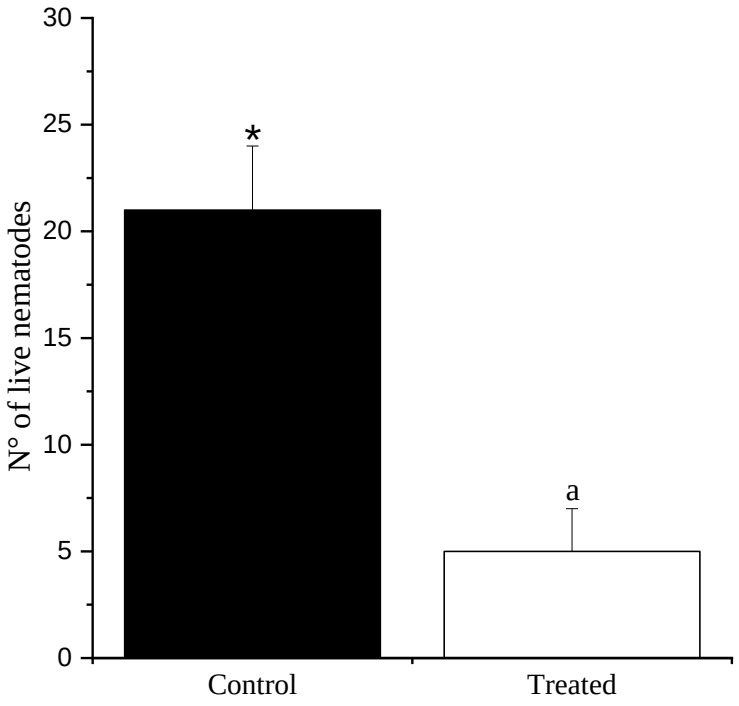

Figure 1: Evaluation of the nematophagous activity of *Pleurotus djamor* against *Trichostrongylus* spp. and, *Strongyloides papillosus* after 6 hours. The letter “a” indicates that there was a significant difference between the treated group and the control ($p<0.01$) by ANOVA at the 1% probability level ($F=142.5$; $DF=11$).

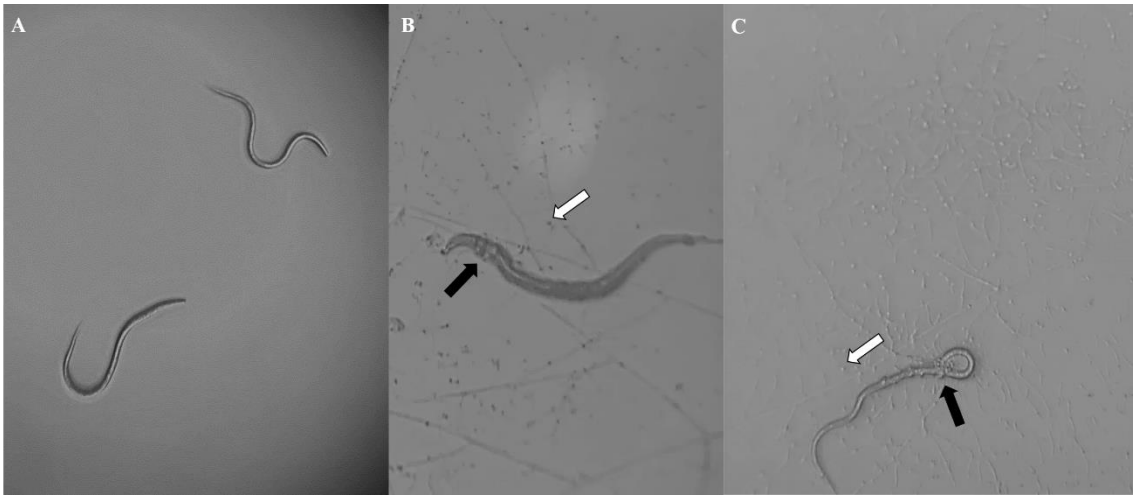

Figure 2: Nematophagous activity of *Pleurotus djamor* against *Trichostrongylus* spp. and, *Strongyloides papillosus*. A: Control group. B e C: Treated. Black arrows: Presence of hyphae intertwined in the nematode's cuticle. White arrows: possible droplets of toxins produced by the mushrooms to paralyze the nematode.

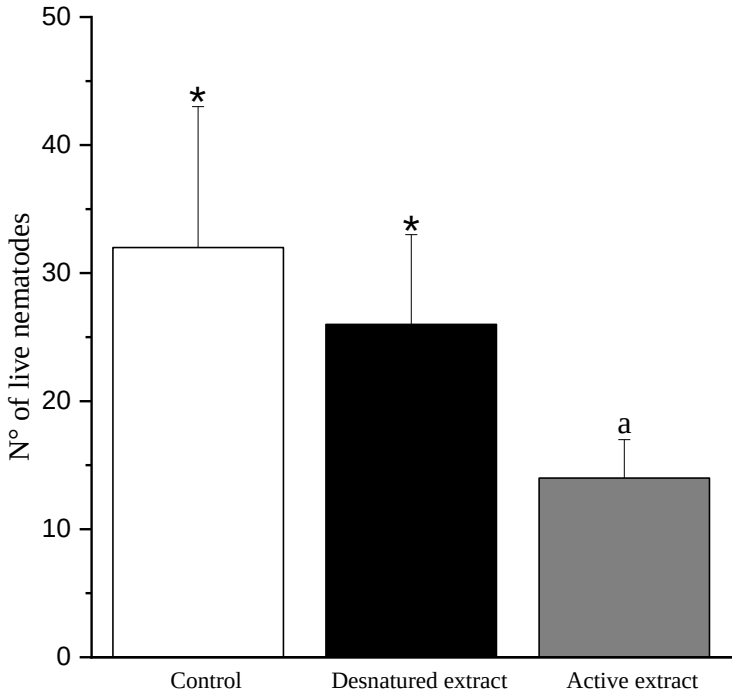

Figure 3: Evaluation of the nematicidal activity of the active crude extracts of the mushroom *Pleurotus djamor* against *Trichostrongylus* spp. and, *Strongyloides papillosus*. after 48 hours. ANOVA data: $p<0.01$; $F=14,64$; $DF=29$. The presence of an asterisk indicates that there was no significant difference between the treated group and the control ($p>0.05$) by Tukey's test at the 1% probability level. The letter “a” indicates that there was a significant difference between the treated group the denatured group and the control ($p<0.01$) by Tukey's test at the 1% probability level.

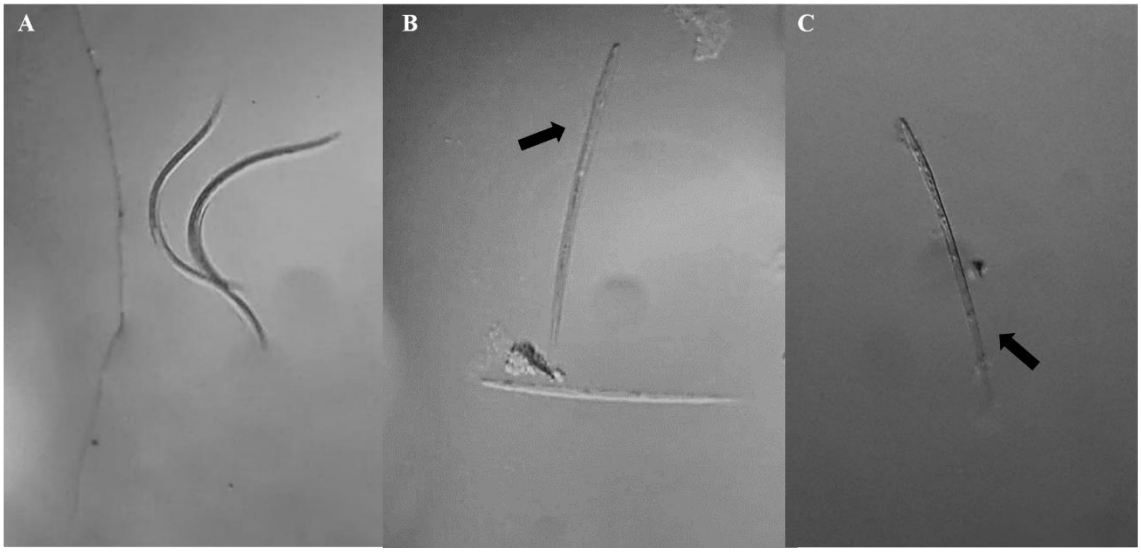

Figure 4: Nematicidal activity of the active crude extract of the *Pleurotus djamor*. A: Control group. B and C: Extract with active enzymes. Black arrows: Paralyzed nematode (Trichostrongylus spp. and, Strongyloides papillosus), and possible start of cuticle degradation.

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5 CAPÍTULO 4

ARTIGO 4:

Use of agricultural waste for optimization of protease production by *Pleurotus djamor* and evaluation of its anthelmintic activity

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COLABORADORES:

Adriane Toledo da Silva[1*], Lisseth Bibiana Puente Figueroa[1], Debora Castro de Souza[1], Dyesse Pollyane Ferreira[1], Pedro Henrique Dutra dos Santos[3], Eustáquio Souza Dias[2], Fabio Ribeiro Braga[3], Filippe Elias de Freitas Soares[1]

1 Department of Chemistry, Laboratory of Biotechnology and Applied Biochemistry, Federal University of Lavras, Brazil

2 Department of Biology, Federal University of Lavras, Brazil

3 Experimental Parasitology and Biological Control Laboratory, Vila Velha University, Brazil

*Corresponding author – filippeufv@yahoo.com.br

ABSTRACT

Pleurotus djamor is an edible mushroom that produces proteases. However, the production of these enzymes needs to be optimized and applied in experimental models. This study aimed to optimize the production of proteases by *Pleurotus djamor* using agricultural waste and to evaluate the anthelmintic activity of proteases *in vitro*. Solid-state fermentation optimized protease production, with wheat bran as the substrate. For this, the central composite design (CCD) was used. The proteolytic activity was determined after the preparation of the cell-free crude extract. In addition, eggs of the helminths *Taenia solium* and *Moniezia* sp. were used to evaluate the *in vitro* anthelmintic activity of the proteases. The results showed that moisture significantly influenced ($p<0.01$) protease production. On the other hand, within the evaluated intervals, the incubation time was non-significant ($p>0.05$). The enzymes led to a significant reduction ($p<0.01$) in *T. solium* and *Moniezi*a sp. eggs, with reductions of 33.44% and 45.43%, respectively, compared to the control with denatured enzymes. The results suggest the action of proteases in the degradation of helminth eggs, demonstrating their potential for biochemistry control.

Keywords: biochemistry control, enzyme, tapeworms, edible mushroom

1. INTRODUCTION

The control of parasitic infections still lacks many studies, especially those aimed at the treatment and salutary measures to control its pre-parasitic forms [1,2]. Thus, among these infections with unique importance in health, the taeniasis-cysticercosis/neurocysticercosis complex caused by *Taenia solium* infection stands out [3,4]. According to WHO (2021), the number of cases is still impressive, both in terms of its magnitude and the difficulty of controlling it in both humans (definitive hosts) and animals, especially pigs (intermediate hosts) [5,6]. On the other hand, another cestode, *Moniezia* sp., although it does not pose major

problems in single health, causes monieziasis in domestic ruminants, leading to significant losses in animal production [7].

Anthelmintics commonly manage these organisms, but their inadequate and isolated use favors the selection of resistant parasites [8,9]. Anthelmintic resistance can be developed through different mechanisms such as parasite genetics, increased metabolism, changing the receptor site of these products which reduces the interaction between the parasite and the anthelmintics applied, among other factors [10].

Faced with this challenge, control alternatives are studied and proposed [11]. For example, the use of aqueous extracts of pomegranate (*Punica granatum*) against eggs of gastrointestinal parasitic nematodes. In this study, the authors achieved 94% inhibition of egg hatching [12]. In addition to organic compounds, the use of enzymes from mushrooms could be a new approach for controlling the eggs of these parasites in the environment, promoting their decontamination and preventing healthy animals from becoming infected [13].

The edible mushroom *Pleurotus djamor* is a basidiomycete that produces protease (EC 3.4), enzymes that catalyze the hydrolysis of peptide bonds in the proteins present in parasite [14]. To produce these enzymes, the culture medium must be optimized. For this purpose, solid-state fermentation (SSF) using residues (e.g., sawdust, vegetable peels, wheat bran) as substrates are widely used [15,16]. Therefore, by reusing and employing agricultural production products, the material that was considered waste is reinserted into the economy. This practice is in line with the principles of the circular economy, which aims for sustainable development based on environmental protection as well as making a profit [17].

Therefore, this study aimed to optimize protease production by the edible mushroom *P. djamor* using agricultural waste and to evaluate the anthelmintic activity of extracellular proteases *in vitro*.

2. MATERIAL AND METHODS

2.1 Protease sourcing and extraction

The edible mushroom *P. djamor* was obtained from the mycoteca collection of the Department of Agricultural Microbiology at the Federal University of Lavras (UFLA). The fungus was kept in Petri dishes containing potato-dextrose-agar 2% (PDA 2%) at 28 °C and in the dark.

The fungal inoculum was prepared using a solid medium according to the modified methodology of Sufiate et al. (2017) from solid-state fermentation (SSF) [13]. The medium was composed of wheat bran residue (washed with boiling water and then dried at 50°C) 5g; K_2HPO_4 5g L^{-1}; $MgSO_4$, 0,1 g L^{-1}; and yeast extract, 5 g L^{-1} at varying moisture levels, depending on the statistical treatment. The medium was placed in Erlenmeyer flasks (50 mL) and autoclaved for 20 minutes at 121°C. Subsequently, three discs (approximately 5mm in diameter) of the mushroom isolate were transferred to each flask, which was kept in the dark at 28°C±1 for time intervals defined by the statistical treatment. Distilled water was added to the flasks in a 1:5 (w/v) ratio to extract the enzymes, and the flasks were shaken at 120 rpm for 1 hour. Then, the suspension was filtered on Whatman No. 4 paper and centrifuged for 10 min at 10,000 g. The supernatant (cell-free crude extract) was collected, and its proteolytic activity was determined [18].

For comparison purposes, submerged fermentation (SmF) was conducted using the culture medium outlined by Genier et al. (2015), with modifications [19]. The liquid medium comprised: Glucose, 10 g L^{-1} K_2HPO_4, 5g L^{-1}; $MgSO_4$, 0,1 g L^{-1}; and yeast extract, 10 g L^{-1}. The volume of 50 mL of the medium was placed in Erlenmeyer flasks (250 mL) and autoclaved for 20 min at 121ºC. Subsequently, three discs (approximately 5 mm in diameter) of the mushroom isolate were transferred to each flask, which was left under agitation at 120 rpm in the dark, at 28°C±1, for seven days. After this period, the extract was filtered on Whatman No.

4 paper and centrifuged for 10 min at 10,000 g. The supernatant (cell-free crude extract) was collected, and its proteolytic activity was determined.

2.2 Proteolytic activity

According to the methodology of Sufiate et al (2017), the proteolytic activity of the cell-free crude extract of *P. djamor* was measured [13]. The conditions used in this assay were: 500 μL of casein (1% w/v) pH 8.0, 400 μL of 50 mM citrate buffer pH 5.0, and 100 μL of cell-free crude extract. The incubation lasted 60 min at 50°C. After this period, one milliliter of trichloroacetic acid (10% v/v) was added to stop the reaction. At the same instant, the trichloroacetic acid and the other components (buffer, casein, and cell-free crude extract) were added in the same concentrations as those mentioned above. The suspension was centrifuged for 10 min at 10,000 g for absorbance reading in a spectrophotometer at 280 nm. The absorbance value obtained after the 60 min reaction was subtracted from the absorbance value found at time zero. Using a tyrosine external standard curve, the enzyme concentration in the extract was calculated. Under these assay conditions, one protease unit was defined as the enzyme required to release 1.0 μg of tyrosine per minute.

2.3 Response surface methodology

Central Composite Design Response, a surface methodology approach, was used in the present study to optimize protease production by the fungus *P. djamor* and to investigate the interaction of the variables time interval (days) and moisture (%). These variables were selected due to their importance in mushroom fermentation and enzyme production [20]. The effects of these variables on the response (protease-proteolytic activity) were studied at five experimental levels: time interval (23.89, 21, 14 (central point), 7 and 4.10 days) and moisture (512.13, 450, 30014 (central point), 150, 87.86%). Thirteen experiments were performed, with five

replications of the central point (Table 1). Minitab® 15 and Design Expert 7.0 (Stat-Ease Inc., USA) software were used for data analysis.

2.4 Obtaining eggs of *Taenia solium* and *Moniezia* sp.

The retrieval of *T. solium* eggs was performed by dissecting proglottids from an adult specimen, obtained from human feces. Immediately afterward, the morphological integrity of the eggs was observed using an optical microscope in the 10x objective [1]. On the other hand, to obtain *Moniezia* sp. eggs, the uterus was dissected from adult parasites from naturally infected sheep from the experimental farm of the Vila Velha University [11].

2.5 Anthelmintic activity

Four experimental groups were defined to evaluate the anthelminthic activity of *P. djamor* on *T. solium* and *Moniezia* sp. eggs. Two groups with active enzymes (AE) and two with denatured enzymes (DE-extract previously boiled for two h). Each group contained 100 µL of the extracts and 100 µL with around 50 and 70 eggs for *T. solium* and *Moniezia* sp., respectively. Six replicates were carried out for each group. After this period, each replicate's number of intact eggs was counted. The count was done using an optical microscope in the 10x objective.

Analysis of variance (ANOVA) was performed on the data obtained in this assay with significance levels of 1 to 5%. Tukey's test evaluated the efficiency of helminth destruction by the groups treated with AE compared to the DE group at 1 to 5 % probability [21]. The following equation was used to calculate the average percentage of reduction in the number of intact eggs [22].

$$\%\text{Reduction} = \frac{\overline{\text{x}}_{\,intact\ eggs\ DE} - \overline{\text{x}}_{\,intact\ eggs\ AE}}{\overline{\text{x}}_{intact\ eggs\ DE}}$$

3. RESULTS

3.1 Response surface methodology

The optimal levels of the two selected variables (incubation time and moisture) for the highest protease production by *P. djamor* were calculated using the CCD and are presented in the complete factorial experimental assay in Table 1. The function for the analyzed response " proteolytic activity", in uncoded terms, after the elimination of non-significant variable terms ($p>0.05$) was:

$$\text{Proteolytic activity} = 45{,}527 - 0{,}23397\ \text{Moisture} + 0{,}00031\ \text{Moisture}^2$$

The data were analyzed using variance analysis and the F test. The quadratic model generated was significant ($P < 0.05$), as shown in Table 2. The correlation coefficient of the model (R^2) was 0.93, demonstrating that the factorial model was significant. Fig. 1 shows the three-dimensional response surface plot obtained from the final model of protease production by the edible mushroom *P. djamor*.

In addition, the maximum proteolytic activity observed in the experimental matrix was 31,61 U mg^{-1} under the following conditions: 14 days of incubation time and 87.86% moisture.

3.2 SSF x SmF

The proteolytic activity obtained from the SmF was compared with the proteolytic activity obtained after optimizing the culture medium using the wheat bran residue (SSF). The corresponding values of proteolytic activity were: 6,92 ± 0,11 and 31,61 ± 2,54 U mL^{-1} for SmF and SSF, respectively. The value of proteolytic activity after optimization was approximately five times higher.

3.3 Anthelmintic activity

The proteases in the AE produced by *P. djamor* showed a significant reduction ($p<0.01$) in the number of intact eggs of *T. solium* and *Moniezia* sp., compared to the DE group, indicating its anthelmintic action *in vitro*. The percentages of reduction, also with the DE group, were: 33.44% (*T. solium*) and 45.43% (*Moniezia* sp.) (Fig. 2).

The proteolytic action of *P. djamor* enzymes can be observed in Fig. 3 and 4. Fig. 3 shows the digestive action of proteases on eggshells and subsequently on the internal content of *T. solium*. In this case, the digestion of the barriers and the egg's contents is clear. Fig. 4 shows an identical action on *Moniezia* sp. eggs. They usually appear triangular (Fig. 4a). However, after the action of proteases, the triangular shape is lost, becoming oval (Fig. 4b). In addition, damage to the eggshells can be observed.

4. DISCUSSION

Our study observed that the proteases produced by the edible mushroom *P. djamor* were important in the digestion of tapeworm eggshells since they have significant amounts of proteins in their composition for the protection of the embryo [11]. Previous studies have reported the anthelmintic activity of proteases. For example, the crude extract of *Pochonia chlamydosporia*, rich in these enzymes, reduced 64.1% ($p<0.01$) of *Ascaridia galli* eggs compared to the control group after 24 hours of incubation [23]. Using the same fungus, it was also observed a 76.8% ($p<0.01$) reduction in the hatching of *A. galli* eggs after 24 hours of incubation [24].

To obtain optimal levels of protease production from *P. djamor*, an optimization strategy known as the Response Surface Methodology was used. This statistical approach uses a quadratic factorial model to determine the maximum levels of variables to achieve a maximum response. In this study, only the linear and quadratic terms of the moisture variable were significant ($p<0.01$) for the evaluated response (proteolytic activity). Therefore, for the model

obtained, none of the variables (incubation time) were significant for protease production. The 3D response surface (Fig. 1) generated after obtaining the quadratic model demonstrates that the lower the moisture value (%), the higher the value of the proteolytic protease activity, regardless of the incubation time.

Previous studies have shown that moisture is an important factor in enzyme production. According to Jatuwong et al. (2020), when using moisture of 85% in the preparation of the phytase-inducing medium by *Pholiota adiposa*, the enzyme showed the highest proteolytic activity. The moisture value used by these authors is close to the result obtained in our study (87.86%). In addition, in that study, they used agricultural residue in the composition of the medium [25].

In this context, Talhi et al. (2022) also used agricultural waste as an inducer medium for protease production by *Mycothermus thermophiles*. Among the different media tested, wheat bran showed the highest proteolytic activity. Moreover, among the various parameters tracked by the authors, it was observed that moisture is one of the significant variables in protease production, in a similar way to this study [26].

For comparison, protease was also produced using SmF in the present study. After optimizing the fermentation process via CCD, the proteolytic activity value using SSF was approximately five times higher. Given these results, the importance of optimizing fermentation processes is remarkable. In this sense, Jatuwong et al. (2020) achieved a phytase activity value 3.15 times higher than the activity before the optimization process [25]. On the other hand, Talhi et al. (2022) obtained a protease activity value 6.17 times higher using the optimal conditions found [26].

Given these promising enzyme activity values obtained in the SSF optimization, some advantages can be mentioned regarding using SmF [27]. SSF stands out for presenting a greater similarity to the natural habitat of the fungus; it has a low cost since the media are prepared

with agricultural residue (wheat bran, oat bran, orange peel, potato peel), lower energy consumption, and also less generation of the waste stream (28-30).

SSF is an efficient method when used as an enzyme inducer medium. Microorganisms can use agro-industrial residues and byproducts as substrates and generate high-value-added products with promising applications [31].

One of these applications is using these enzymes to control zoonotic parasite [32]. The present study used the protease-rich AE produced by *P. djamor* to control *T. solium* and *Moniezia* sp. The diseases caused by these parasites are a public health concern, as they are considered neglected diseases [33, 34].

To control these parasites, proteases have relevant advantages over anthelmintics since their presence in soil or water does not pose a risk of contamination for both the environment and humans [35]. In addition, using alternatives to control *T. solium* and *Moniezia* sp. is interesting in periods of shortage of conventional anthelmintics.

During this period, the AE could be used as a complement to control parasites in the environment, thus avoiding the indiscriminate use of anthelmintics and respecting the safe dose of these products that may be present in the environment and food. In addition, it would also prevent anthelmintic resistance [10,33,37].

The use of alternatives to anthelmintics in the control of *Moniezia* sp. has been reported previously. Braga et al. (2008) used the fungus *Paecilomyces lilacinus* to control this tapeworm [1]. They obtained a maximum reduction percentage of 23% ($p<0.01$) after 15 days of incubation of the eggs together with the fungus. In the present study, using *the P. djamor* AE, a higher reduction percentage of 45.43% ($p<0.01$) was achieved after 24 hours of incubation. As observed by the authors, morphological alterations were observed in the eggshell (Fig. 4).

In this context, Araújo et al. (2010) also used the fungus *P. lilacinus* to control *T. saginata* eggs [38]. After 15 days of incubation, they obtained a 24.6% reduction. On the other

hand, in the present study, the protease-rich AE was evaluated on *T. solium* eggs, and a higher reduction percentage of 33.44% was obtained. In both studies, egg destruction was observed (Fig. 3).

5. CONCLUSION

This study suggests that *P.djamor* protease-rich AE, after optimization of the medium with agricultural residue (wheat bran) at 87.86 % moisture, has potential in the biochemical control of *T. solium* and *Moniezia* sp. Therefore, further research needs to be carried out to study the application of this protease-rich AE in environments contaminated by eggs of these parasites.

Author's contributions

Silva Adriane: Conceptualization, Methodology, Investigation, Formal analysis, Writing - Original draft preparation, Writing - Review and Editing. Figueroa Lisseth: Investigation, Writing- Review and Editing. Souza Debora: Investigation, Writing- Review and Editing. Ferreira Dyesse: Investigation, Writing- Review and Editing. Santos Pedro: Investigation, Writing- Review. Dias Eustáquio: Resources, Writing- Reviewing and Editing. Ribeiro Fábio: Writing- Reviewing and Editing. Soares Filippe: Conceptualization, Methodology, Supervision, Writing- Original draft preparation, Writing - Review and Editing.

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Data availability

Not applicable.

Declarations

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Competing interests

The authors declare no competing interests.

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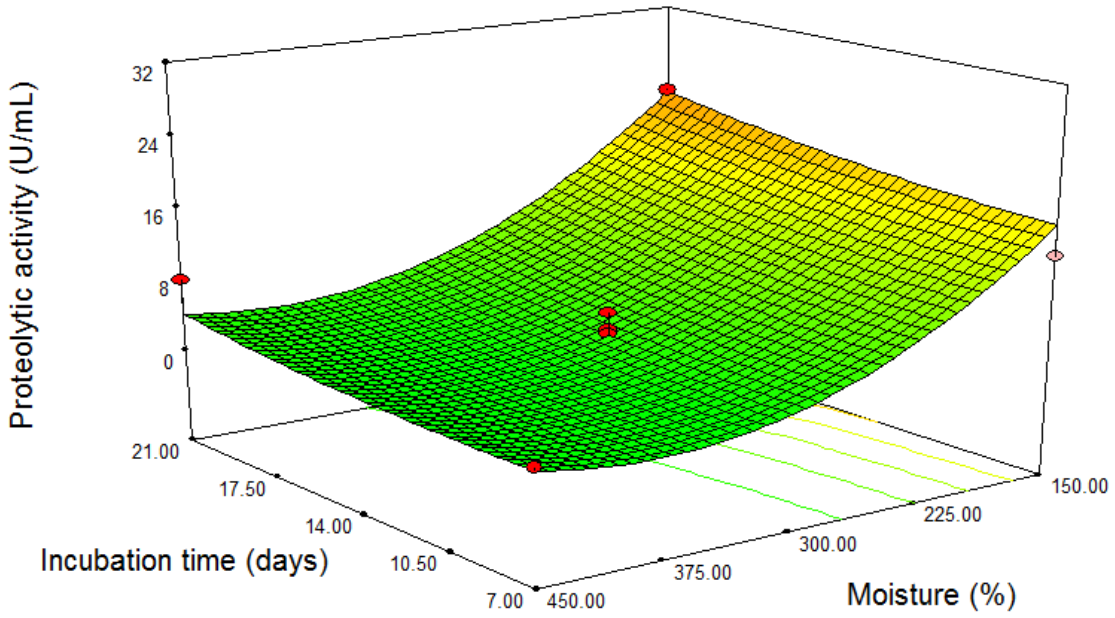

Fig. 1 Response surface curve of protease production by the edible mushroom *Pleurotus djamor* in solid-state fermentation using agro-industrial residue (wheat bran).

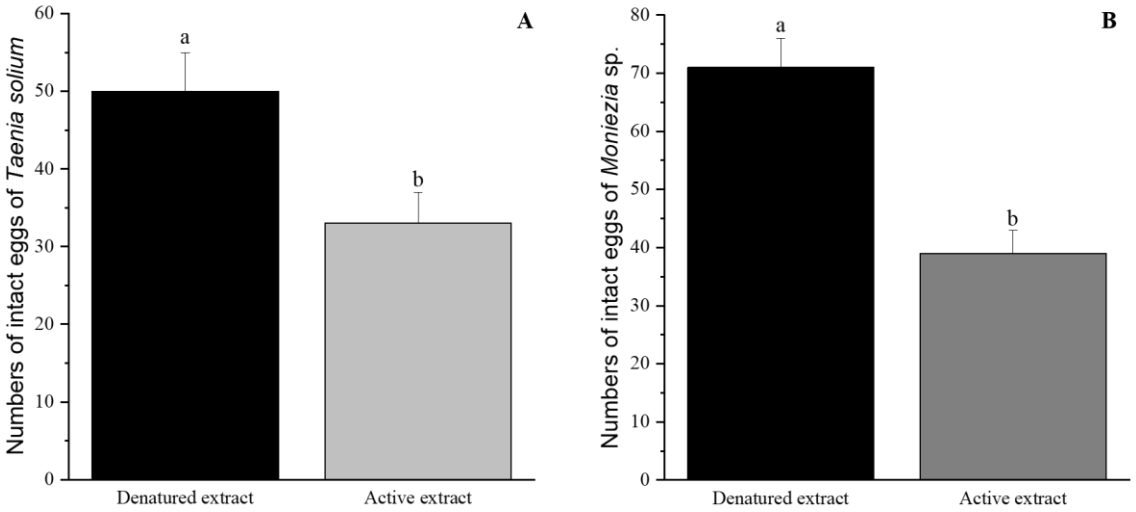

Fig. 2 Evaluation of the anthelminthic activity of *Pleurotus djamor* proteases on *Taenia solium* and *Moniezia* sp. after 24 hours of incubation at 28±1°C, in the dark. Different letters indicate that the control group with denatured extract and the group treated with the active extract for both parasites showed a significant difference ($p<0.01$).

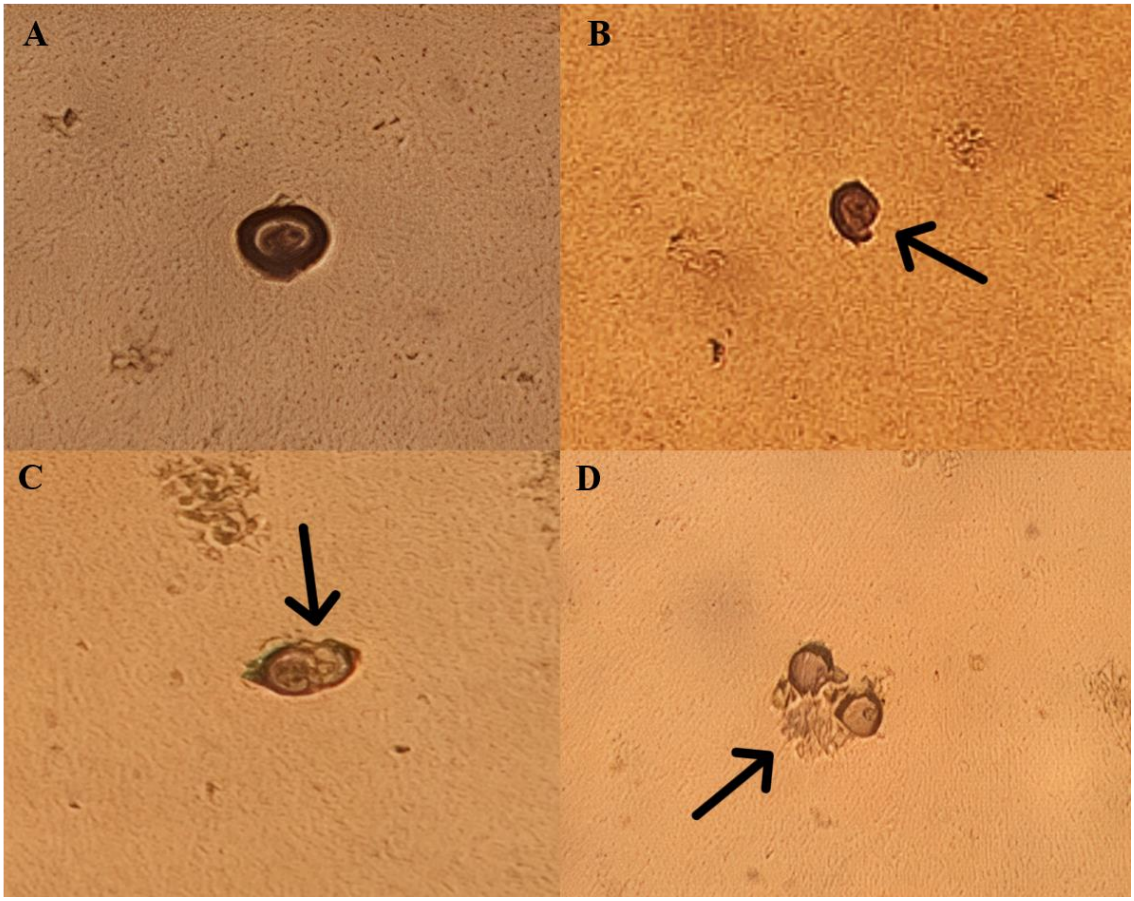

Fig. 3 Antiparasitic activity of *Pleurotus djamor* protease on *Taenia solium* eggs *in vitro*. A: Denatured extract. B, C, and D: Active extract. Black arrows indicate the digestion of the shell and internal contents due to the action of proteases.

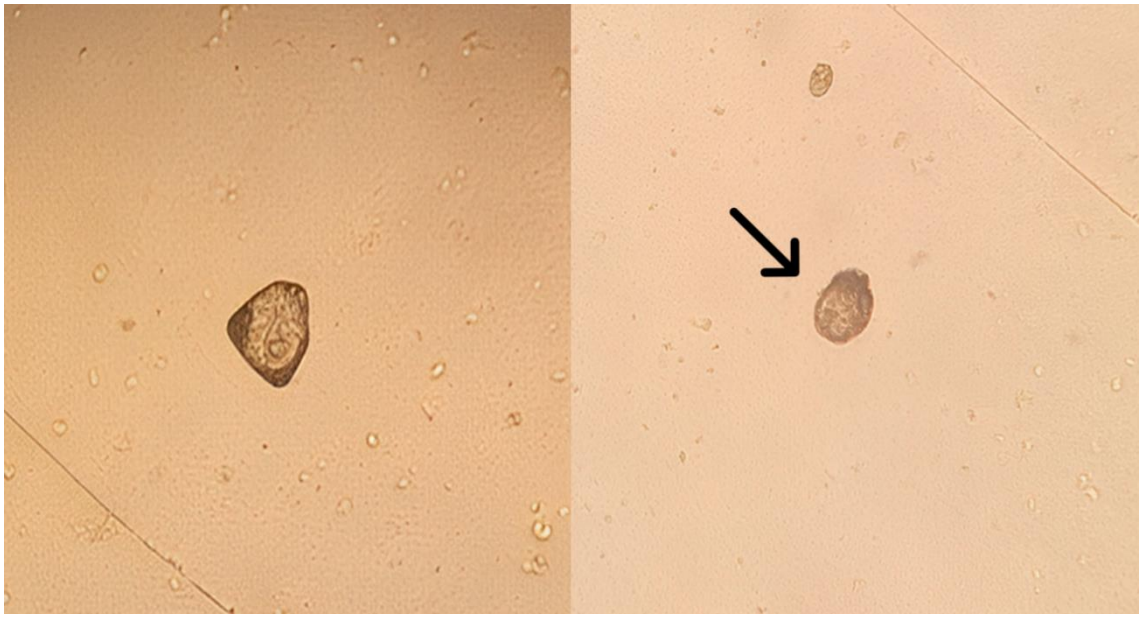

Fig. 4 Antiparasitic activity of *Pleurotus djamor* protease on *Moniezia* sp., eggs *in vitro*. A: Denatured extract. B: Active extract. The black arrow indicates the digestion of the shell by the action of proteases; there was a change in the morphology of the egg.

Table 1: Experimental design with 13 points for applying the response surface methodology using a Central Composite Design with two variables (incubation time and moisture) and the values of the analyzed response (proteolytic activity) for each point.

Runs	Incubation time (days)	Moisture (%)	Proteolytic activity (U/mL)
1	7	150	13.92
2	21	150	22.17
3	7	450	2.07
4	21	450	7.99
5	4.10	300	4.89
6	23.89	300	4.88
7	14	87.86	31.61
8	14	512.13	2.77
9	14	300	2.44
10	14	300	4.08
11	14	300	3.63
12	14	300	6.02
13	14	300	1.26

Table 2: Analysis of variance (ANOVA) for the response equation developed for protease production by the edible mushroom *Pleurotus djamor*. The units of the variables incubation time and moisture were days and percentages, respectively.

Source	P>F
Model	0.0005
Incubation time	0.1464
Moisture	0.0001
Incubation time*Moisture	0.7150
$(\text{Incubation time})^2$	0.5008
Moisture	0.0005

6 CAPÍTULO 5

ARTIGO 5:

Proteolytic profile and nematicidal potential of proteases produced by *Pleurotus djamor* in coprocultures

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COLABORADORES:

Adriane Toledo da Silva[1*], Debora Castro de Souza[1], Lisseth Bibiana Puentes Figueroa[1], Amanda do Carmo Alves[1], Tiago Moreira Facury[2], Eustáquio Souza Dias[3], Fábio Ribeiro Braga[4], Filippe Elias de Freitas Soares[1]

1 Laboratório de Biotecnologia e Bioquímica Aplicada, Departamento de Química, Universidade Federal de Lavras, Lavras – MG, Brasil

2 Departamento de Clínica e Cirurgia Veterinária, Escola de Veterinária, Universidade Federal de Minas Gerais, Belo Horizonte – MG, Brasil

3 Departamento de Biologia, Universidade Federal de Lavras, Lavras – MG, Brasil

4 Laboratório de Parasitologia Experimental e Controle Biológico, Universidade Vila Velha, Vila Velha – ES, Brasil

*Corresponding author – filippe.soares@ufla.br

ABSTRACT

The aim of this study was to evaluate the proteolytic profile of the cell-free crude extract (CFCE) of *Pleurotus djamor* and its nematicidal action on *Haemonchus* spp. and *Trichostrongylus* spp. larvae in coprocultures. Solid-state fermentation was used to produce proteases by *P. djamor*. The proteolytic and specific activity of CFCE was measured, and its proteolytic profile was revealed using a zymogram. The nematicidal assay of CFCE in coprocultures was evaluated using two groups: the control group (G1) containing 1 mL of CFCE with denatured enzymes + 10 g of positive feces, and the treated group (G2) containing 1 mL of active CFCE + 10 g of positive feces. For each group, 10 repetitions were carried out. A proteolytic activity of 7.5 ± 0.9 U mL^{-1} and a specific activity of 30 U mg^{-1} were measured. With regard to the nematicidal assay, there was a significant 35% reduction in the number of larvae recovered from G2 compared to G1. The zymogram showed the presence of at least two proteases in the active CFCE of *P. djamor* with molecular weights between 75 and 100 kDa. The results suggest that the CFCE of *P. djamor* has nematicidal potential even in the feces environment.

Keywords: enzymes, mushroom, *Haemonchus* spp., *Trichostrongylus* spp.

The economic losses caused by gastrointestinal nematodes (GINs) are incalculable (Liu et al., 2023). These parasites are mainly controlled using anthelmintics (levamisole, closantel, and moxidectin). However, excessive use favors the selection of individuals resistant to these control agents (Bordes et al., 2020; Hao et al., 2024). In this context, the search for sustainable and effective control strategies has become the subject of much research (Anjos et al., 2024).

The literature points to some studies that highlight enzymes as alternatives in the biochemical control of GINs, especially those that belong to the hydrolase class, since they play a fundamental role in the process of degrading the proteins present in their cuticle (Soares et

al., 2019). Recently, Silva et al. (2024a) highlighted the production of cell-free crude extract (CFCE) rich in proteases (EC 3.4) by the fungus *Pleurotus djamor* for the in vitro control of GINs. However, there are no reports on the nematicidal action of *P. djamor* CFCE in the fecal environment.

Therefore, the aim of this study was to evaluate the proteolytic profile of *P. djamor* CFCE and its nematicidal action on *Haemonchus* spp. and *Trichostrongylus* spp. larvae in coprocultures.

The isolate of *P. djamor* used in all the experiments carried out in this study was supplied by the Fungal Culture Collection of the Laboratory of Edible Mushrooms, Department of Biology at the Federal University of Lavras (UFLA) (Silva et al., 2024b). The mushrooms were kept under refrigeration and in the dark on 2% w/v potato-dextrose-agar (PDA) plates.

According to the modified methodology of Sufiate et al. (2017), solid-state fermentation was employed for the production of protease-rich CFCE by *P. djamor*. The medium was prepared in 125-mL Erlenmeyer flasks with 5 g of wheat bran (previously washed with boiling water and dried at 50 °C) and 4.4 mL of a solution containing K_2HPO_4 5 g L^{-1}, $MgSO_4$ 0.1 g L^{-1}, and yeast extract, 5 g L^{-1}. The flasks were kept in the dark at 28 °C ± 1 for 14 days. The proteolytic activity of *P. djamor* was measured according to Silva et al. (2024b). The assay conditions were: 50 °C, 0.5 M citrate buffer pH 5, 60 min incubation. Under these conditions, one unit of protease enzyme activity was defined as the amount of enzyme required to release 1 µg of tyrosine per minute. The Bradford methodology was used to measure the concentration of soluble proteins in the CFCE (Bradford et al., 1976). The specific activity was then calculated by dividing the protease activity value by the soluble protein concentration.

The proteolytic profile of the CFCE of *P. djamor* was evaluated by zymogram using a 10% polyacrylamide gel containing copolymerized casein (casein-SDS-PAGE). The formation

of white halos was the result of the action of proteases present in the CFCE (Braga et al., 2012; Nagarathnam et al., 2010).

For the coprocultures, feces were collected directly from the rectum of sheep naturally infected with *Haemonchus* spp. and *Trichostrongylus* spp. from the Department of Veterinary Clinic and Surgery of the Federal University of Minas Gerais, Minas Gerais, Brazil. All the fecal material was homogenized, and the EPG (eggs per gram of feces) test was carried out in triplicate according to Gordon and Whitlock (1934), with an average value of 7900 eggs/g.

Ten grams of fecal material was used to prepare the coprocultures (Mohamed et al., 2023), establishing two experimental groups: a control group with denatured CFCE, previously boiled for 2 hours (G1) and a group treated with active CFCE (G2). The groups contained 10 replicates. One milliliter of denatured CFCE was added to G1, and 1.0 mL of active CFCE was added to G2. After 7 days, *Haemonchus spp*. and *Trichostrongylus spp*. larvae were recovered and counted (Roberts and O'Sullivan, 1950).

The data obtained was interpreted using the Bioestat 5.0 statistical program, using analysis of variance (ANOVA) and the Tukey mean test, at significance levels of 1 to 5% probability (Ayres et al., 2003). The average percentage reduction of larvae was calculated using the equation below (Mendoza De Gives et al., 1994):

$$\%\text{Reduction} = \frac{\overline{\text{x}}_{L_3\ (\text{CFCE desnaturado})} - \overline{\text{x}}_{L_3\ (\text{CFCE ativo})}}{\overline{\text{x}}_{L_3\ (\text{CFCE desnaturado})}}$$

In the present study, the active CFCE had a proteolytic activity of 7.5 ± 0.9 U mL^{-1} and a specific activity of 30 U mg^{-1}. On the other hand, denatured CFCE showed no proteolytic activity.

The results showed that the number of larvae recovered from the group treated with active CFCE (G2) showed a significant difference ($p<0.01$) compared to the number of larvae recovered from the control group with denatured CFCE (G1). In addition, there was a 35%

reduction in the number of larvae recovered. Therefore, it is suggested that the active enzymes present in the CFCE of the G2 group were effective in reducing the number of larvae in the fecal sample (Fig.1).

This study is a pioneer in evaluating the effect of proteolytic enzymes from *P. djamor* on GINs in coprocultures. Recently, Silva et al. (2024a) investigated the in vitro nematicidal effect of active CFCE on *Trichostrongylus* spp. and *Strongyloides pappilosus* larvae, achieving a 75% significant reduction ($p<0.01$) in the number of larvae. However, the present study demonstrated the applicability of enzymes in controlling helminths even in a fecal environment, since active CFCE reduced 35% of larvae in coproculture.

The literature addresses the in vitro action of *P. djamor* as a nematicide due to its ability to produce toxins, such as allitol and terpene, obtaining significant results with a reduction percentage of 92.56% (González-Cortázar et al., 2021). However, this study points to a complementary approach to the control of GINs through the production of active CFCE from *P. djamor*, i.e., without the use of mycelia of the fungus and directly on feces environment. Therefore, the potential for controlling larvae excreted in deposited animal feces was demonstrated here, an application proposal that differs from the majority of studies reported in the literature, which propose the control of these parasites in the digestive tract of ruminants (Braga et al., 2020; González-Cortázar et al., 2021).

As the results show, the use of active CFCE from *P. djamor* is promising because, after the 7-day evaluation period, it showed significant nematicidal action. Thus, it can be suggested that, even under the inhospitable conditions of the fecal environment, with a high microbial concentration, pH, humidity, and saline concentration that were not optimal, the enzymes maintained their biological activity. Similarly, the study by Soares et al. (2015) observed the same stability of the proteases when using the active CFCE produced by *Monacrosporium thaumasium* to control *Angiostrongylus vasorum* in coprocultures.

In addition, in a recent study by Souza et al. (2024), proteases produced by the nematophagous fungus *Duddingtonia flagrans* demonstrated nematicidal action on *Haemonchus* spp. and *Trichostrongylus* spp. larvae in coprocultures. The authors observed a 36% reduction in the number of nematodes when using the active CFCE of *D. flagrans* compared to the control group. This result was similar to the 35% reduction obtained in the present study, which used CFCE rich in active proteases from *P. djamor*. This mushroom is also a nematophagous fungus, but it belongs to the toxin-producing fungi group, while *D. flagrans* belongs to the nematode-trapping group.

The zymogram is an approach that, in addition to confirming enzymatic activity, can provide a proteolytic profile of the CFCE (Dell Valle et al., 2023) (Fig. 2). The casein in the gel acts as a substrate. The region where the casein hydrolysis reaction occurs due to the proteolytic action of the enzymes is not stained. Thus, after staining and discoloration of the gel, a white halo can be seen due to casein digestion (Cho et al., 2020) (Fig. 2). The bands indicated on the zymogram suggest the presence of at least two proteases in the active CFCE of *P. djamor* with a molecular weight between 75 and 100 kDa, as indicated by the molecular marker. A similar result was reported by Santana et al. (2024), who evaluated the proteolytic profile of the active CFCE of *P. eryngii* and the authors observed the presence of at least two proteases between 75 and 100 kDa.

The protease-rich CFCE from *P. djamor* showed promising results in controlling GIN larvae in coprocultures. In addition, the proteolytic profile suggested the presence of at least two proteases in the CFCE. Thus, the present study indicates that *P. djamor* produces proteases with potential nematicidal action even in a fecal environment.

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CRediT authorship contribution statement

Silva Adriane: study design, methodology, investigation, formal analysis, writing of original draft preparation, review, and editing. **Souza Debora**: investigation, review, and editing. **Figueroa Lisseth**: investigation, review, and editing. **Alves Amanda**: investigation, review, and editing. **Facury Tiago**: resources, review, and editing **Dias Eustáquio**: resources, review, and editing. **Braga Fábio**: writing, review, and editing. **Soares Filippe**: study design, methodology, supervision, writing of original draft preparation, review, and editing.

Declaration of competing interest

None.

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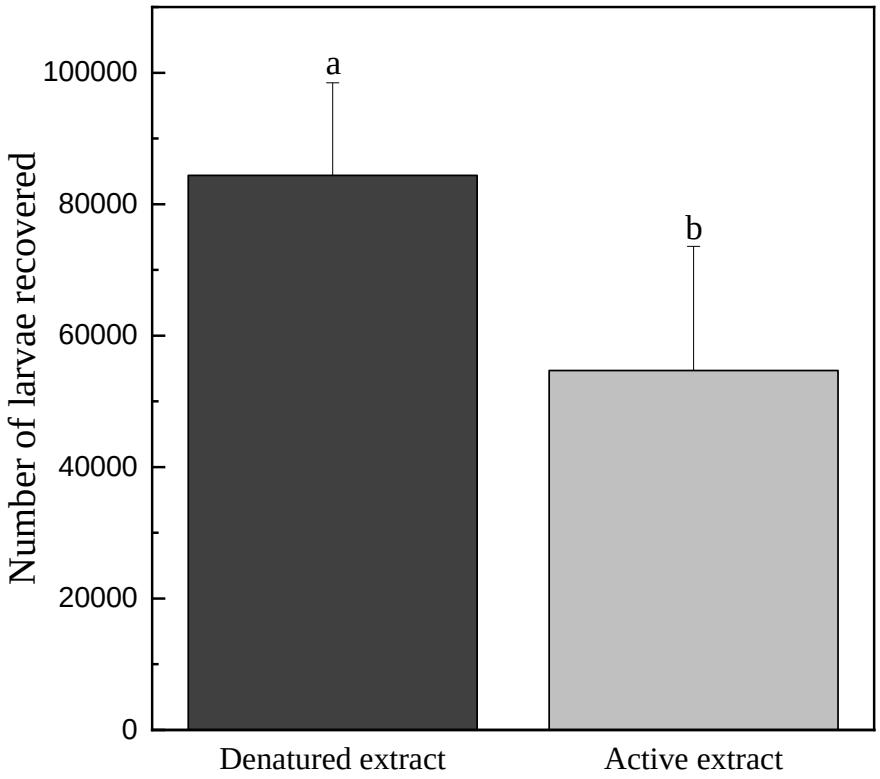

Fig.1. Nematicidal effect of proteases from the cell-free crude extract (CFCE) of *Pleurotus djamor* on *Haemonchus* spp. and *Trichostrongylus* spp. larvae in coprocultures. Different letters indicate that there was a significant difference between the denatured extract and the active extract ($p<0.01$).

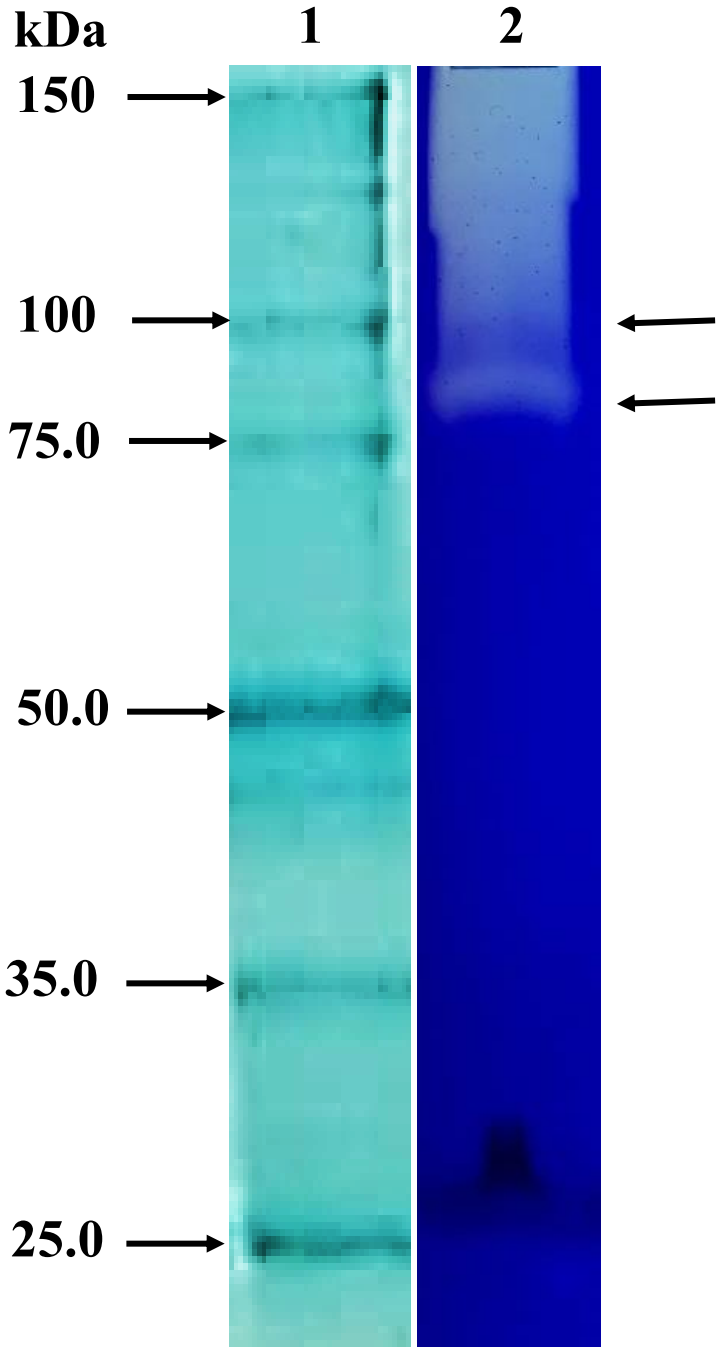

Fig.2. Zymogram of the cell-free crude extract (CFCE) from *Pleurotus djamor*. 1: Molecular mass marker. 2: CFCE from *P. djamor*. Black arrows indicate the proteases present in the native PAGE gel (10%) with casein (1% m/v).

7 CAPÍTULO 6

ARTIGO 6:

Nematicidal activity of *Pleurotus djamor* metabolites on *Meloidogyne incognita* under greenhouse conditions

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Nematicidal activity of *Pleurotus djamor* metabolites on *Meloidogyne incognita* under greenhouse conditions

Atividade nematicida de metabólitos de *Pleurotus djamor* sobre *Meloidogyne incognita* em casa de vegetação

Actividad nematicida de los metabolitos de *Pleurotus djamor* sobre *Meloidogyne incognita* en condiciones de invernadero

Adriane Toledo da Silva
PhD student in agrochemistry
Training institution: Federal University of Lavras
Address: Lavras, Minas Gerais, Brasil
E-mail: adrianetoledo123@gmail.com

Dyesse Pollyane Ferreira
Undergraduate in agronomy
Training institution: Federal University of Lavras
Address: Lavras, Minas Gerais, Brasil
E-mail: dyesse.ferreira@estudante.ufla.br

Debora Castro de Souza
PhD student in agrochemistry
Training institution: Federal University of Lavras
Address: Lavras, Minas Gerais, Brasil
E-mail: debora.souza7@estudante.ufla.br

Júlia Carvalho de Araújo
Undergraduate in agronomy

Training institution: Federal University of Lavras
Address: Lavras, Minas Gerais, Brasil
E-mail: julia.araujo4@estudante.ufla.br

Ana Carolina Silva
Master student in agrochemistry
Training institution: Federal University of Lavras
Address: Lavras, Minas Gerais, Brasil
E-mail: ana.silva157@estudante.ufla.br

Eustáquio Souza Dias
PhD in Genetics and Breeding.
Training institution: Federal University of Lavras
Address: Lavras, Minas Gerais, Brasil
E-mail: esdias@ufla.br

Willian César Terra
PhD in Agronomy (Phytopathology)
Training institution: Federal University of Lavras
Address: Lavras, Minas Gerais, Brasil
E-mail: willianterra@ufla.br

Filippe Elias de Freitas Soares
PhD in Agricultural Biochemistry
Training institution: Federal University of Lavras
Address: Lavras, Minas Gerais, Brasil
E-mail: filippe.soares@ufla.br

ABSTRACT

This study evaluated the nematicidal activity of the cell-free crude extract (CFCE) of *Pleurotus djamor* on *Meloidogyne incognita* under greenhouse conditions. The fungus was grown in a solid medium for 14 days in the dark. Distilled water was added to the medium, filtered, and centrifuged, and the proteolytic activity of the CFCE produced was measured. Three experimental groups were established: control group (distilled water), CFCE (denatured enzymes), and CFCE (active enzymes). The nematicidal activity of the extracts was assessed in vitro on second-stage juveniles (J2) and eggs of *M. incognita*, separately. Under greenhouse conditions, CFCEs were applied to tomato seedlings grown on substrate infested with *M. incognita* eggs. About the in vitro tests, the active CFCE showed a significant difference concerning the control group ($p<0.05$) according to the t-test, with a 22% reduction in eggs. On the other hand, about J2, both treatments showed a significant difference compared to the control ($p<0.01$) with a 100% reduction, but no significant difference between themselves ($p>0.05$). Similarly, the treatments in the greenhouse showed the same statistical behavior as the control ($p<0.01$). However, there was no significant difference between them ($p>0.05$) regarding the number of eggs and the number of galls. The reduction in the number of eggs was 94 and 97 % and in the number of galls was 71 and 67% for denatured and active CFCE, respectively. This study points to the nematicidal action of metabolites on J2 and the activity of enzymes on *M. incognita* eggs.

Keywords: Biochemical control. Nematicidal activity. Metabolites. Mushroom.

RESUMO

Este estudo avaliou a atividade nematicida do extrato bruto livre de células (CFCE) de *Pleurotus djamor* sobre *Meloidogyne incognita* em condições de estufa. O fungo foi cultivado em um meio sólido por 14 dias no escuro. Em seguida, a atividade proteolítica do CFCE produzido foi medida. Foram estabelecidos três grupos experimentais: grupo de controle (água destilada), CFCE (enzimas desnaturadas) e CFCE (enzimas ativas). A atividade nematicida dos extratos foi avaliada in vitro em juvenis de segundo estágio (J2) e ovos de *M. incognita*, separadamente. Em condições de estufa, os CFCEs foram aplicados a mudas de tomate cultivadas em substrato infestado com ovos de *M. incognita*. Em relação aos testes *in vitro*, o CFCE ativo apresentou uma diferença significativa em relação ao grupo de controle ($p<0,05$) de acordo com o teste t, com uma redução de 22% nos ovos. Por outro lado, em J2, ambos os tratamentos apresentaram uma diferença significativa em relação ao controle ($p<0,01$) com uma redução de 100%, mas nenhuma diferença significativa entre si ($p>0,05$). Os tratamentos na estufa apresentaram o mesmo comportamento estatístico que o controle ($p<0,01$). No entanto, não houve diferença significativa entre eles ($p>0,05$) com relação ao número de ovos e ao número de galhas. A redução no número de ovos foi de 94 e 97% e no número de galhas foi de 71 e 67% para CFCE desnaturado e ativo, respectivamente. Este estudo aponta para a ação nematicida dos metabólitos em J2 e a atividade das enzimas em ovos de *M. incognita*.

Palavras-chave: Controle bioquímico. Atividade nematicida. Metabólitos. Cogumelo.

RESUMEN

En este estudio se evaluó la actividad nematicida del extracto crudo libre de células (CFCE) de *Pleurotus djamor* sobre *Meloidogyne incognita* en condiciones de invernadero. El hongo se cultivó en un medio sólido durante 14 días en la oscuridad. Se añadió agua destilada al medio, que luego se filtró y centrifugó, y se midió la actividad proteolítica del CFCE producido. Se establecieron tres grupos experimentales: grupo de control (agua destilada), CFCE (enzimas desnaturalizadas) y CFCE (enzimas activas). La actividad nematicida de los extractos se evaluó in vitro sobre juveniles de segundo estadio (J2) y huevos de *M. incognita*, por separado. En condiciones de invernadero, los CFCE se aplicaron a plántulas de tomate cultivadas en sustrato infestado con huevos de *M. incognita*. En relación con los ensayos in vitro, los CFCE activos mostraron una diferencia significativa respecto al grupo control ($p<0,05$) según la prueba t, con una reducción del 22% de los huevos. Por otro lado, con respecto al J2, ambos tratamientos mostraron una diferencia significativa con respecto al control ($p<0,01$) con una reducción del 100%, pero ninguna diferencia significativa entre ellos ($p>0,05$). Del mismo modo, los tratamientos en el invernadero mostraron el mismo comportamiento estadístico en relación con el control ($p<0,01$). Sin embargo, no hubo diferencias significativas entre ellos ($p>0,05$) en cuanto al número de huevos y al número de agallas. La reducción en el número de huevos fue del 94 y 97 % y en el número de agallas fue del 71 y 67% para CFCE desnaturalizado y activo, respectivamente. Este estudio apunta a la acción nematicida de los metabolitos sobre J2 y a la actividad de las enzimas sobre los huevos d*e M. incognita*.

Palabras clave: Control bioquímico. Actividad nematicida. Metabolitos. Hongo.

1 INTRODUCTION

Agriculture is heavily affected by plant-parasitic nematodes, especially those of the genus *Meloidogyne* spp., which are known as root-knot nematodes. Among the species of root-knot nematodes, the most important is *M. incognita*, with more than 3000 plant species affected by this phytopathogen (Jones et al., 2013; Liang et al., 2020). Crops infected by these phytonematodes have lower yields of around 5 to 15% compared to healthy crops. This loss is equivalent to 150 billion dollars (Khan, 2023). Among the vegetables affected by *M. incognita* parasitism, tomatoes are one of the most damaged (Oliveira et al., 2019; Panno et al., 2021).

Controlling phytonematodes is generally complicated. Nowadays, nematode control is predominantly made using chemical molecules and genetic resistance, although biological control methods are also employed (Sikora et al., 2018). However, chemical nematicides are potentially harmful to the environment (Shilpa et al., 2022; Xue et al., 2024). Actually, the most efficient chemical nematicides have been removed from the market (Wram and Zasada, 2019). In view of this, new alternatives are essential for more efficient and environmentally friendly control (Desaeger et al., 2020; Khan et al., 2024).

In this context, *Pleurotus djamor* is an edible mushroom with excellent nutritional value and nematophagous action that has been recognized for decades (Hibbett and Thorn, 1994). This fungus is also a source of secondary metabolites and enzymes with a nematicidal effect (Gómez-Rodrígues et al., 2023; Pineda-Alegria et al., 2024; Silva et al., 2024). Among the enzymes, proteases (EC 3.4) stand out as hydrolytic enzymes that act as catalysts in the degradation reaction of proteins present in the cuticle and eggs of *M. incognita* (Soares et al., 2023). Therefore, the aim of this study was to evaluate the proteolytic activity of the cell-free crude extract (CFCE) of *P. djamor* on *M. incognita* in vitro and under greenhouse conditions.

2 METHODOLOGY

2.1 ORGANISMS

The isolate of *P. djamor* used in this study was obtained from the Fungal Culture Collection of the Laboratory of Edible Mushrooms, Department of Biology at the Federal University of Lavras (UFLA). *P. djamor* was kept in Petri dishes containing 2% potato-dextrose agar (PDA2%) under refrigeration and in the dark.

For the production of proteases by *P. djamor*, solid-state fermentation was used, according to the modified methodology of Sufiate et al. (2017). The medium was prepared using 5 g of wheat bran (previously washed with boiling water and dried at 50 °C), 4.4 mL of K_2HPO_4, 5 g L^{-1}, $MgSO_4$, 0.1 g L^{-1}, and yeast extract, 5 g L^{-1}. The medium was transferred to

Erlenmeyer flasks (50 mL) and autoclaved for 30 minutes at 121 °C. After cooling, three disks with a diameter of approximately 5 mm obtained from *P. djamor* plates were added to each Erlenmeyer flask. The flasks were then placed in an incubator and kept in the dark at 28 °C±1 for 14 days.

2.2 ENZYMATIC ASSAYS

The proteases were extracted using distilled water in a solid/liquid ratio of 1:5 (w/v). To do this, the flasks were placed in a shaker and stirred at 120 rpm for 1 hour. After this stage, the aqueous suspension was filtered through Whatman N°. 4 paper and centrifuged at 10,000 g for 10 min to obtain the protease-rich CFCE.

The proteolytic activity of the *P. djamor* CFCE was then measured under the following conditions: 50 °C, 60 min, and pH 5 (50 mM citrate buffer). After reading in a spectrophotometer at 280 nm, one unit of protease was defined as the amount of enzyme needed to release 1 µg of tyrosine per minute (Silva et al., 2024).

2.3 *Meloidogyne incognita*

A pure population of *M. incognita* was multiplied in tomato seedlings (*Solanum lycopersicum* L. 'Santa Clara') kept in a greenhouse for 60 days. After this period, the *M. incognita* eggs were extracted from the tomato roots using the methodology proposed by Hussey and Barker (1973). For the experiments with J2, the egg suspension was placed in a hatching chamber and incubated at a temperature of 28 °C. The hatched J2 used for the assays was collected between 48 and 72 hours after the hatching chamber was set up. To calibrate the suspension, the J2s were transferred to glass slides and quantified under a microscope (Whitehead and Heming, 1965).

2.4 *IN VITRO* EFFECT OF *Pleurotus djamor* CELL-FREE CRUDE EXTRACT (CFCE) ON SECOND-STAGE JUVENILE (J2) OF *Meloidogyne incognita*

After extracting the eggs, the solution was cleaned. Twelve grams of kaolin were added to the tube containing around 40 mL of the egg solution. The suspension was then taken to the centrifuge for 5 min at 300 g. The supernatant was discarded, and a 45% (w/v) sucrose solution was added to the pellet, and the tube was shaken to homogenize it. Again, the solution was taken to the centrifuge for 1 min at 200 g. The supernatant was poured into a 500-mesh sieve. The eggs retained on the sieve were transferred to a 15-mL Falcon tube. The eggs were counted using an optical microscope with a 10x objective (Coolen and D'Herde, 1972).

The assay was carried out using a methodology similar to that of the previous assay. However, 30 µL of an aqueous suspension containing around 60 *M. incognita* eggs plus 30 µL of active or denatured CFCE were added to each 200 µL microtube. As a negative control, 30 µL of distilled water and approximately 60 eggs were used. Six replicates were carried out for each treatment. The microtubes were then kept in an incubator at 28 °C±1 for 72 hours. After this period, the intact eggs were counted using an optical microscope with a 10x objective (Sufiate et al., 2017).

2.5 *Pleurotus djamor* CELL-FREE CRUDE EXTRACT (CFCE) ON SUBSTRATES INFESTED WITH *Meloidogyne incognita* EGGS

Tomato plants with 2 pairs of leaves were transplanted into plastic pots filled with 85 g of vegetable substrate (Tropstrato®, Vida Verde Indústria e Comércio de Insumos Orgânicos Ltda., Mogi Mirim, São Paulo, Brazil). Then, the pots with the tomato seedlings (one seedling in each pot) were taken to the greenhouse, where they remained for 11 days. After this period, the substrate was infested. To do this, four holes were made in the substrate around the tomato stem. Next, 250 µL of an aqueous suspension containing around 75 *M. incognita* eggs were added to each hole. In this way, each pot received around 300 eggs. Then, 250 µL of the solution containing the active CFCE or the denatured extract was poured into each hole. As a negative control, only 250 µL of distilled water and *M. incognita* eggs were used. Six replicates were made for each treatment. After the treatments were applied, the pots were returned to the greenhouse, where they remained for 77 days. After this period, the following parameters were measured: stem diameter, root length, length from the root to the first leaf, and the number of galls. Finally, the eggs were extracted, as described above.

2.6 STATISTICAL ANALYSIS

The bioassay data were initially subjected to the Shapiro-Wilk and Bartlett tests to assess normality and homoscedasticity, which are necessary conditions for conducting analysis of variance (ANOVA). After this verification, a one-way ANOVA was performed using the BioEstat software version 5.3 to evaluate the differences between treatment groups. When ANOVA indicated significant differences, Tukey's test and the t-test were applied for pairwise comparisons of means. Statistical significance was set at $p<0,05$ and $p<0,01$, respectively (Ayres et al., 2003). The average percentage reduction in the number of galls and eggs obtained in this assay was calculated using the equation (1) described by Mendoza-De Gives e Vazquez-Prats (1994):

$$\%\text{Reduction} = \frac{\overline{x}_{\text{eggs or galls (control group)}} - \overline{x}_{\text{eggs or galls (active CFCE)}}}{\overline{x}_{\text{eggs or galls (control group)}}} \quad (1)$$

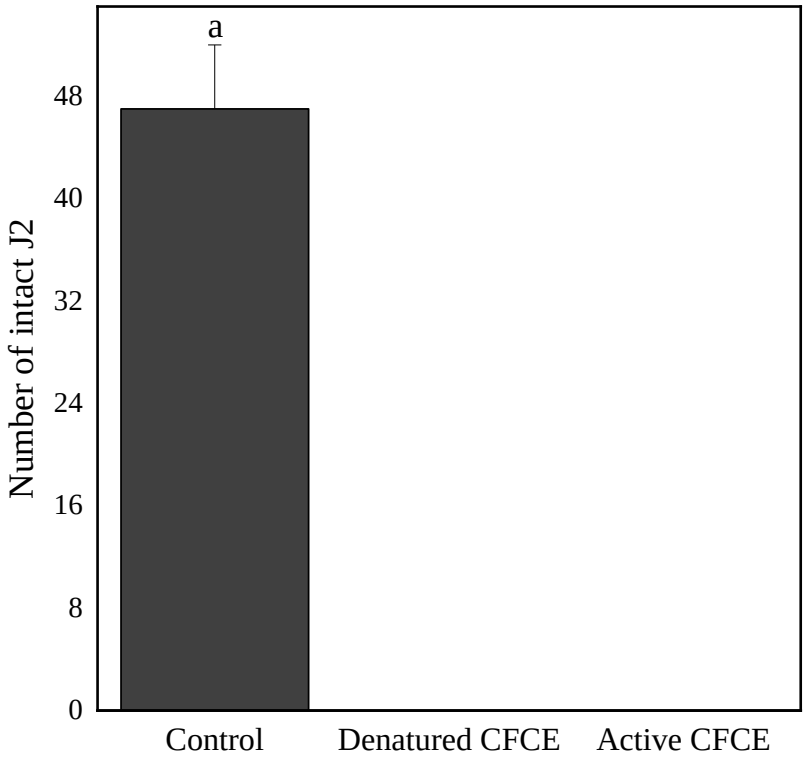
3 RESULTS AND DISCUSSIONS

This study used solid-state fermentation to produce CFCE with a proteolytic activity of 34 ± 2 U mL^{-1}. The study by Sufiate et al. (2017) used the same culture medium and obtained a proteolytic activity of 32.74 U mL^{-1}, similar to that found in the present study.

Previously, Silva et al. (2024) used a solid culture medium to induce protease production by *P. djamor*, consisting of wheat seeds, to obtain CFCE and showed an enzymatic activity of 8.3 ± 0.2 U mL^{-1}, i.e., four times lower compared to the results obtained in the present study. In view of this, it was noted that the culture medium used in this study was better for inducing protease production by *P. djamor*.

In the present study, both the CFCE with active proteases and the denatured crude extract showed high ($p<0.01$) nematicidal activity on the J2 of *M. incognita* (Figure 1). Surprisingly, the denatured CFCE showed no significant difference from the active CFCE ($p>0.05$). The percentage reduction of J2 was 100% for both groups, compared to the control group.

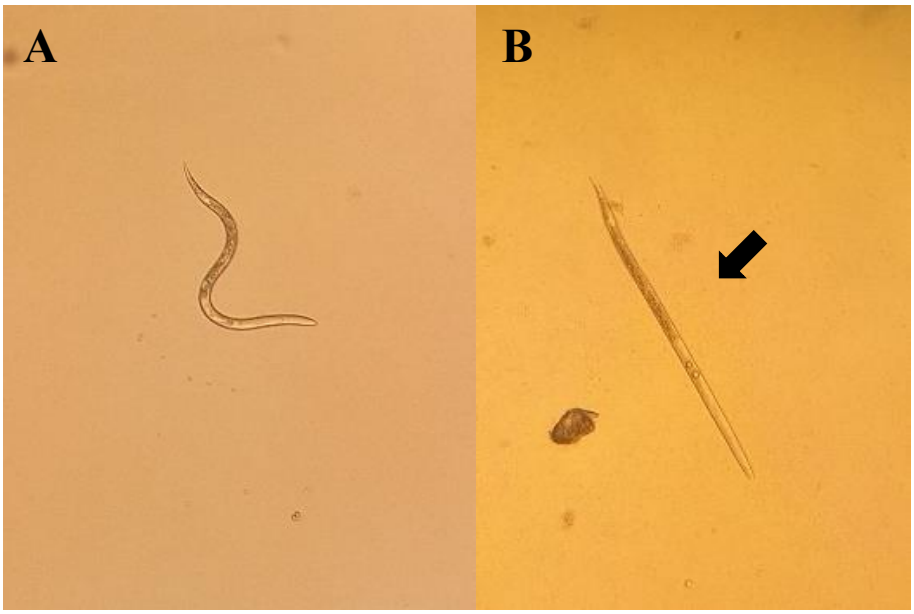
Figure 1. In vitro nematicidal activity of the active and denatured cell-free crude extract (CFCE) from *Pleurotus djamor* on second-stage juveniles (J2) of *Meloidogyne incognita*. The conditions of the assay were: 24 hours of incubation, 28±1°C, in the dark. Letter a: Indicates that the treated groups showed a significant difference compared to the control group ($p<0.01$). Bars denote the standard deviation.



Source: Prepared by the authors themselves.

The immobile and dead J2 can be seen in Figure 2 due to the effect of the metabolites and enzymes present in the CFCE.

Figure 2. Nematicidal effect of the cell-free crude extract (CFCE) of *Pleurotus djamor* on second-stage juveniles (J2) of *Meloidogyne incognita*. A: Control group. B: Group treated with active CFCE from *Pleurotus djamor*. The black arrow highlights the immobile and dead nematode.



Source: Prepared by the authors themselves.

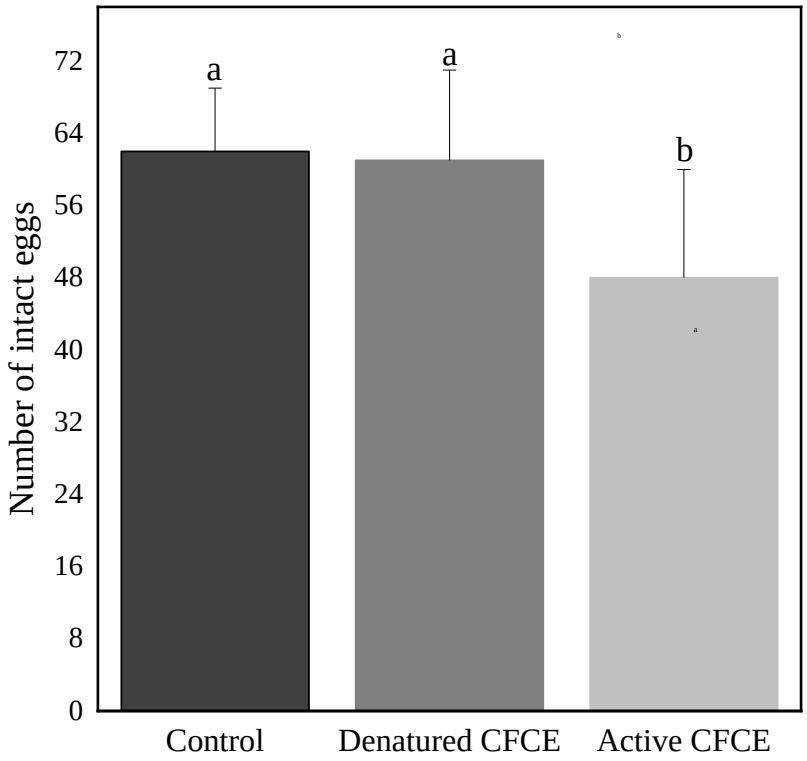
In a recent study, Silva et al. (2024) demonstrated that the enzymes from *P. djamor* had a nematicidal effect on *Panagrellus* sp. In that study, the active CFCE showed a significant difference compared to the denatured CFCE ($p<0.01$). Furthermore, no significant difference was observed between the control group and the denatured group ($p>0.05$). The enzyme-rich CFCE showed a percentage reduction of 73%. However, in this study, the nematicidal effect (Figure 2) was probably not due solely to the action of the proteases, since the denatured CFCE also killed 100% of the nematodes. In addition to enzymes, CFCE has metabolites with nematicidal activity (Sufiate et al., 2017).

It was previously described in the literature by Hibbett and Thorn (1994) that *Pleurotus* spp. produce toxins derived from linoleic acid. Later, Pineda-Alegria et al. (2017) identified four fatty acids and a terpene in the hydroalcoholic extract of *P. djamor*, namely: pentadecanoic, hexadecanoic, octadecadienoic, octadecanoic acid, and β-sitosterol. Fungi of this genus use these toxins as a destructive mechanism against nematodes (Castañeda-Ramírez et al., 2020).

The results of the in vitro assay using active and denatured CFCE on J2 of *M. incognita* showed similar effects to those observed by Sufiate et al. (2017) who used active and denatured CFCE from *P. eryngii* on *Panagrellus* sp. Sufiate et al. (2017) also showed that the groups treated with active CFCE did not differ significantly from the groups treated with denatured CFCE ($p<0.01$). In addition, those authors observed reduction percentages of 90 and 91 % for active and denatured CFCE, respectively. On the other hand, in the present study the percentage reduction was 100% for both treatments (Figure 1).

Figure 3 shows the in vitro assay results for the control group, denatured and active CFCE on *M. incognita* eggs after 72 hours. The control group (water) showed no significant difference from the group treated with CFCE containing denatured enzymes ($p>0.05$). However, the group treated with active CFCE showed a significant difference compared to the control group and the group with denatured CFCE ($p<0.05$). The percentage reduction of intact *M. incognita* eggs treated with active CFCE was 22% in comparison to the control group.

Figure 3. Evaluation of the in vitro nematicidal activity of the active and denatured cell-free crude extract (CFCE) of *Pleurotus djamor* on *Meloidogyne incognita* eggs after 72 hours of incubation at 28±1°C in the dark. Different letters: Indicate that the group with active CFCE showed a significant difference compared to the control group and the group with denatured CFCE ($p<0.01$). Equal letters: Indicate that the group with denatured CFCE and the control group showed no significant difference ($p>0.05$). Bars denote the standard deviation.



Source: Prepared by the authors themselves.

In this same experiment, the presence of live hatched J2 was only observed in the control group with water. In the group treated with denatured CFCE, some J2 also hatched, but all were dead. These results are in line with those previously observed in the in vitro assay using

denatured CFCE on J2 (Figure 1). On the other hand, the group treated with active CFCE did not have any hatched J2. These results strongly suggest the catalytic activity of the enzymes in the degradation of proteins from eggshell of *M. incognita* (Figure 4). Furthermore, it could be suggested that the enzymes affected the J2 hatching.

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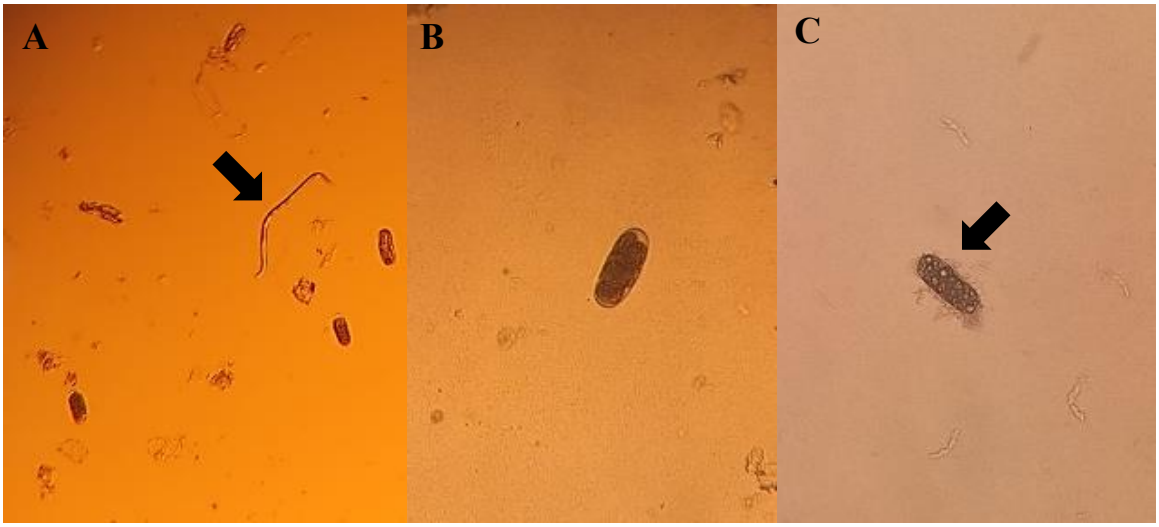
Figure 4. In vitro nematicidal effect of proteases from the active cell-free crude extract (CFCE) of *Pleurotus djamor* on *Meloidogyne incognita* eggs. A: Control group. B: Denatured CFCE. The black arrow indicates the presence of juveniles in the control group (A) and digestion in the eggshell resulting from protease activity in the group with active CFCE (C).



Source: Prepared by the authors themselves.

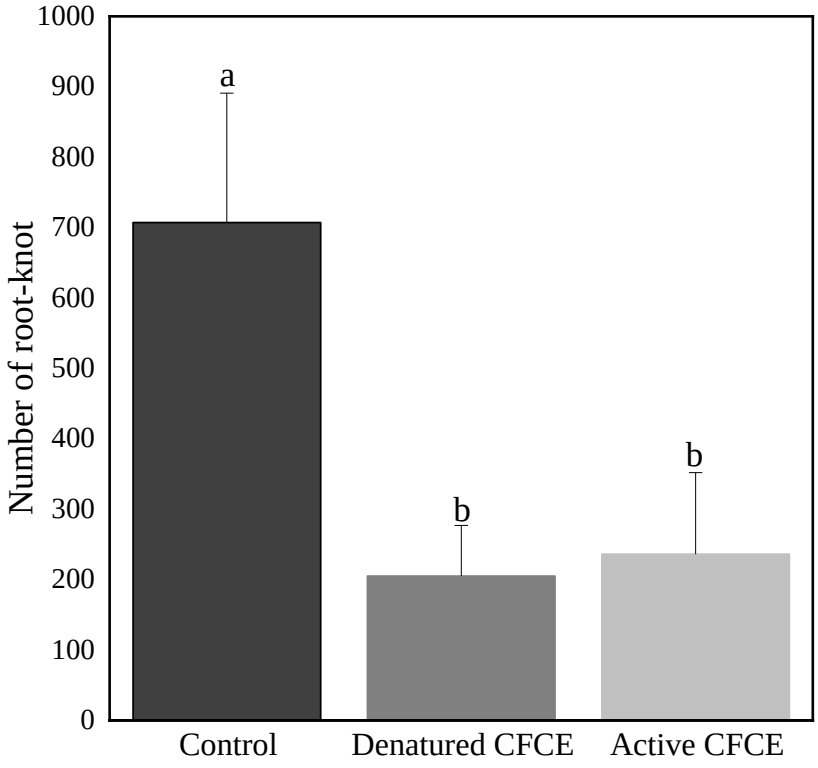
Similarly, the effect of active and denatured CFCE on *M. incognita* eggs observed in this study was similar to the experiment using *Meloidogyne* spp. eggs described by Sufiate et al. (2017) where there was also a significant difference ($p<0.01$) between the groups treated with CFCE with denatured and active enzymes. Those authors obtained an egg reduction percentage of 53% compared to the denatured group. In the present study, the percentage of egg reduction was 22% for the CFCE group with active enzymes compared to the control group (water) (Figure 3).

Meloidogyne spp. eggs have a shell composed of three layers: an inner layer rich in lipids, an intermediate layer composed of chitin-protein complexes and an outer layer formed by lipoproteins (Liang et al., 2020). This constitution is favorable for the hydrolysis of the proteins present in the eggshell through the action of proteases. In this context, the eggs will be damaged or destroyed, impairing the J2 hatching (Khan et al., 2004; Xu et al., 2021), as

observed in this study. Furthermore, this is the first report presenting the effect of *P. djamor* proteases on *M. incognita* eggs.

Regarding the results of the greenhouse assay, the groups treated with active and denatured enzymes reduced the number of galls and *M. incognita* eggs obtained from the roots of tomato plants ($p<0.01$), compared to the control group (water). However, there was no significant difference ($p>0.05$) between them. With regard to the number of galls, the percentage reductions were 71 and 67% for the denatured and active CFCE, respectively, compared to the control group (water) (Figure 5).

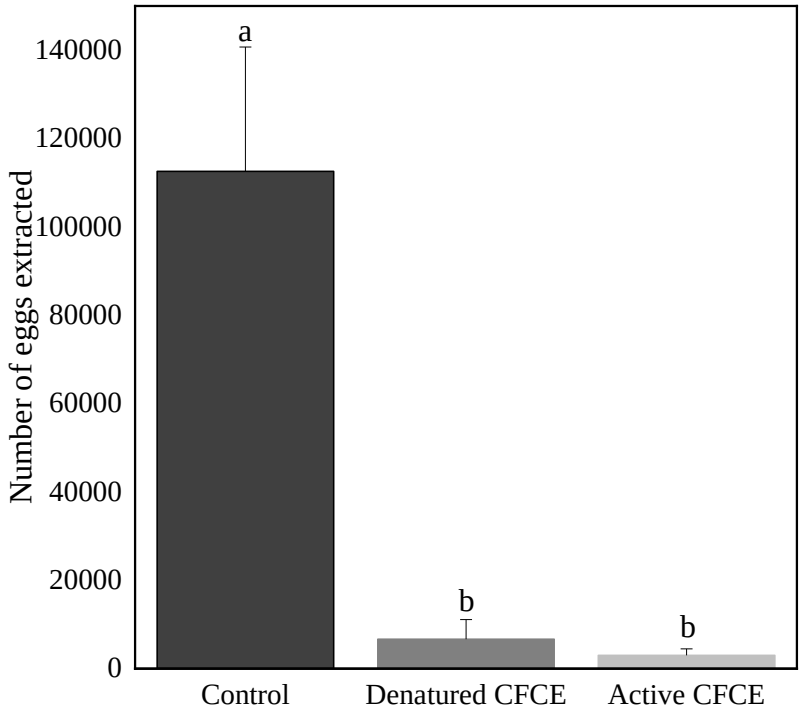
Figure 5. Number of galls on the roots of *Solanum lycopersicum* in the control group and in the groups treated with active and denatured cell-free crude extract (CFCE) of *Pleurotus djamor* after 77 days under greenhouse conditions. Different letters: Indicate that the treated groups showed a significant difference compared to the control group ($p<0.01$). Equal letters indicate that the denatured and active CFCE groups showed no significant difference ($p>0.05$). Bars denote the standard deviation.



Source: Prepared by the authors themselves.

Regarding the number of eggs, the percentage reductions were 94 and 97 % for denatured and active CFCE, respectively (Figure 6).

Figure 6. Number of *Meloidogyne incognita* eggs extracted from the roots of *Solanum lycopersicum* in the control group and in the groups treated with active and denatured cell-free crude extract (CFCE) of *Pleurotus djamor* after 77 days under greenhouse conditions. Different letters indicate that the treated groups showed a significant difference compared to the control group ($p<0.01$). Equal letters indicate that the denatured and active cell-free crude extract (CFCE) groups showed no significant difference ($p>0.05$). Bars denote the standard deviation.



Source: Prepared by the authors themselves.

In this sense, when measuring stem diameter, root length and length from the root to the last leaf, there was no difference ($p>0.05$) between the three groups (control, denatured and active). These results reinforce the advantages of using CFCE from *P. djamor t*o control eggs and J2 of *M. incognita*, since CFCE had no impact on the development of *S. lycopersicum*.

On the other hand, the study by Machado (2019) reported that the spent mushroom substrate (SMS) of *P. djamor* favors plant development. In agreement, Lopes et al. (2023) observed that SMS promoted the development of Vera lettuce roots. In addition, it is important to note that according to these authors, using concentrations of 15 to 30% of *P. djamor* SMS in lettuce cultivation does not cause any phytotoxic effect on its development. However, Lopes et al. (2023) point out that higher concentration of the extract can cause phytotoxicity, which is in line with this study.

4 CONCLUSION

This study is the first to report the proteolytic action of CFCE from *P. djamor* on *M. incognita* eggs. The in vitro experiment showed that the proteases in active CFCE accelerate the degradation of eggshell proteins, inhibiting hatching. The authors suggest that, in addition to proteases, the toxins present in CFCE also played an important role in reducing J2 and eggs and *M. incognita* in vitro and under greenhouse conditions. These results indicate the potential of CFCE as a sustainable alternative to the chemical control of these phytoparasites, benefiting agriculture and promoting more ecological practices. However, due to the limited scale of the study, carried out only in controlled environments such as in vitro and greenhouses, the generalization of the results is restricted. Therefore, future studies are recommended to isolate the toxins present in CFCE and test their efficacy in field conditions and against different nematode species.

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8 CAPÍTULO 7

ARTIGO 7:

Atividade nematicida *in vitro* de *Pleurotus djamor* e seu extrato proteolítico associados à papaína

FORMATO: Research paper

PERIÓDICO: Revista Brasileira de Ciências Agrárias

STATUS: Submetido

FATOR DE IMPACTO: 0.4

CITESCORE: 0.6

Atividade nematicida *in vitro* de *Pleurotus djamor* e seu extrato proteolítico associados à papaína

Adriane Toledo Batista da Silva[1*], Ana Carolina Silva[1], Amanda do Carmo Alves[1], Nivia Kelly Lima Sales[1], Eustáquio Souza Dias[2], Filippe Elias de Freitas Soares[1]

1 Departamento de Química, Laboratório de Biotecnologia e Bioquímica Aplicada, Universidade Federal de Lavras

2 Departamento de Biologia, Universidade Federal de Lavras

*Corresponding author – filippe.soares@ufla.br

Abstract

Nematode management requires effective and environmentally safe control methods. This study aimed to evaluate the nematicidal activity of combining *Pleurotus djamor* or its proteolytic extract with papain against *Panagrellus* sp. Additionally, the enzymatic stability and inhibition of this combination were assessed. *In vitro* assays evaluated the fungus or its extract, combined with papain (1% w/v), against the nematodes at 2 and 24 hours. Assays for proteolytic stability and inhibition using PMSF (phenylmethylsulfonyl fluoride) were also conducted. All data were analyzed using ANOVA followed by Tukey's test. The fungus combined with papain reduced the number of nematodes by 45% (at 2 hours) and 92% (at 24 hours). The extract combined with papain yielded reductions of 29% (2 hours) and 32% (24

hours). In contrast, after 24 hours, papain alone reduced nematodes by 73%, while the extract alone achieved a 40% reduction. The proteolytic activity of the combination dropped to zero within 24 hours, indicating potential cross-degradation, and PMSF completely inhibited the activity, confirming the presence of serine proteases in the extract. The association did not exhibit a synergistic effect. Ultimately, the fungal extract limits the long-term efficacy of papain due to mutual proteolytic degradation, a novel finding that must be considered in the development of sustainable biological formulations.
Keywords: Edible mushroom, enzyme, synergism, compatibility, *Panagrellus* sp.

Atividade nematicida *in vitro* de *Pleurotus djamor* e seu extrato proteolítico associados à papaína

Resumo

O manejo de nematoide demanda métodos de controle eficazes e ambientalmente seguros. No presente estudo objetivou-se avaliar a atividade nematicida da combinação de *Pleurotus djamor* ou de seu extrato proteolítico com a papaína sobre *Panagrellus* sp., além disso avaliou a estabilidade enzimática e inibição da combinação. Em ensaios *in vitro*, testou-se o fungo ou extrato combinados com papaína (1% m/v) sobre os nematoides por 2 e 24 horas. Realizaram-se ensaios de estabilidade proteolítica e inibição por PMSF (fenilmetilsulfonilfluoreto). Todos os ensaios foram analisados via ANOVA e teste de Tukey. O fungo com papaína reduziu os nematoides em 45% (2 h) e 92% (24 h). O extrato com papaína reduziu os nematoides em 29% (2 h) e 32% (24 h); após 24 h, a papaína isolada reduziu os nematoides em 73% e o extrato, 40%. A atividade proteolítica da combinação zerou em 24 h, indicando possível degradação cruzada, e o PMSF inibiu totalmente a atividade, confirmando a presença de serino proteases no extrato. A associação não produz efeito sinérgico. O extrato fúngico limita a eficácia de longo prazo da papaína devido à degradação proteolítica mútua, resultado inédito que deve ser considerado no desenvolvimento de formulações biológicas sustentáveis.
Palavras-chave: cogumelo comestível, enzima, sinergismo, compatibilidade, *Panagrellus* sp.

INTRODUCTION

The presence of nematodes of agricultural and veterinary importance remains one of the most persistent challenges to the productivity and sustainability of production systems (Mendoza-De Gives, 2022). In crop production, these parasites are associated with significant economic losses on a global scale, estimated at approximately $80 billion annually, while also compromising the efficiency of water, nutrients, and agricultural inputs (Abd-Elgawad, 2024; Vasantha-Srinivasan et al., 2025). In animal production, particularly concerning small ruminants, gastrointestinal nematodes also generate substantial health and economic impacts, with well-documented losses in sheep farming (Chagas et al., 2022; Faria et al., 2025). Against this backdrop, the development of control alternatives that are simultaneously effective, accessible, and environmentally safer has become a critical need (Chen et al., 2026).

Biological and biochemical control based on fungi and proteolytic enzymes has garnered increasing attention. Fungi of the genus *Pleurotus* exhibit recognized nematophagous potential, either through toxin production or the secretion of proteases (EC 3.4), enzymes that catalyze the hydrolysis of peptide bonds in proteins present in the nematode cuticle (Silva et al., 2024a). In parallel, plant proteases, such as papain, have shown promising nematicidal activity due to their ability to also catalyze the degradation of proteins in these organisms, compromising their physical integrity and survival (Castro et al., 2023; Njom et al., 2021). The study of these biomolecules aligns with the search for more sustainable inputs that are compatible with integrated management strategies and the growing need to reduce agrochemical use (Soares et al., 2023).

Despite these advances, the available literature has predominantly focused on evaluating nematicidal agents in isolation (Ayaz et al., 2024). Consequently, it remains largely unexplored whether the combination of different proteolytic sources can enhance, preserve, or compromise the nematicidal effect observed when these agents are applied separately (Bhat et al., 2023). The successful development of biopesticides depends not only on proving the individual biological activity of their components but also on ensuring their compatibility, maintaining their activity over time, and understanding potential biochemical interactions that may affect final performance (Mawcha et al., 2025).

Given this context, the objective of this study was to evaluate the nematicidal activity of combining the fungus *P. djamor*, or its cell-free crude extract, with papain. Additionally, this study aimed to assess the enzymatic stability of this combination and investigate the inhibition profile of the proteases present in the samples using phenylmethylsulfonyl fluoride (PMSF).

MATERIAL AND METHODS

Microorganisms and nematodes

The edible mushroom isolate *P. djamor* used in the experiments was obtained from the Fungal Culture Collection of the Edible Mushroom Laboratory, Department of Biology, Federal University of Lavras (UFLA). *Pleurotus djamor* was cultured in Petri dishes containing 2% (w/v) Potato Dextrose Agar (PDA) and stored under refrigeration in the dark (Silva et al., 2024b). The free-living nematodes (*Panagrellus* sp.) were maintained on oat-and-water-based culture media in Petri dishes at the Laboratory of Biotechnology and Applied Biochemistry, Department of Chemistry, UFLA (Sufiate et al., 2017).

Papain sourcing and *Pleurotus djamor* protease production

The papain used in this study was acquired commercially (ACS Científica). The production of *P. djamor* enzymes was carried out via solid-state fermentation (SSF) in 125-mL Erlenmeyer flasks containing 5 g of wheat bran (previously washed with boiling water and oven-dried at 50 °C) and 4.4 mL of a nutrient solution composed of K_2HPO_4 (5 g/L), $MgSO_4$ (0.1 g/L), and yeast extract (5 g/L). This followed the moisture parameters and optimized methodology described by Silva et al. (2025).

After 14 days of cultivation, the entire content of the flasks (substrate + fungus) was transferred to 250-mL Erlenmeyer flasks for the extraction step. Distilled water was added at a ratio of 1:5 (w/v) relative to the mass weight substrate + fungus. The mixture was kept under agitation at 180 rpm at 25 ± 1 °C for 1 hour to ensure the extraction of extracellular enzymes into the liquid medium. Subsequently, the material was filtered using filter paper and centrifuged at 10,000 × *g* for 10 min to obtain the cell-free crude extract, in which the proteolytic activity was determined.

Proteolytic assay

Proteolytic activity was evaluated using the caseinolytic assay. The procedure followed the optimized conditions described by Silva et al. (2024a), consisting of 100 µL of sample: G1 (*P. djamor* cell-free extract), G2 (1% w/v papain), and G3 (*P. djamor* cell-free extract + 1% w/v papain), incubated at 50 °C for 60 min at pH 5 (citrate buffer). One enzymatic unit (U) was defined as the amount of protease required to release 1 µg of tyrosine per minute. To quantify the tyrosine in the extract, a calibration curve was plotted using an external tyrosine standard, covering concentrations from 20 to 160 $\mu g \cdot mL^{-1}$ (Soares et al., 2019).

Nematicidal assays

The first nematicidal assay evaluated the effect of the developing mycelium of *P. djamor* combined with papain. This assay was conducted using four experimental groups: G1 (control group), G2 (treated with *P. djamor*), G3 (treated with 1% w/v papain), and G4 (treated with *P. djamor* + 1% w/v papain). To perform this assay, Petri dishes containing water agar (2% w/v) were prepared. Groups G2 and G4, which contained the fungus, were previously inoculated for 10 days and maintained in a BOD incubator in the dark at 28 ± 1 °C. Each experimental group consisted of eight replicates. The experiment was conducted at two distinct time intervals: 2 hours and 24 hours.

For the 2 hours assay, a suspension containing approximately 2,000 *Panagrellus* sp. individuals per 100 µL was used. For the 24 hours assay, a suspension with about 500 *Panagrellus* sp. individuals per 100 µL was employed. The following was added to each experimental group: G1—100 µL of the nematode suspension + 100 µL of distilled water; G2—100 µL of the nematode suspension + 100 µL of distilled water; G3 and G4—100 µL of the nematode suspension + 100 µL of the 1% (w/v) papain solution. The plates were transferred to an incubator and kept at 28 ± 1 °C for the established time intervals. The nematodes were recovered using the Baermann method and counted under a light microscope using a 10× objective (Souza et al., 2023).

The second nematicidal assay was performed using four experimental groups: G1 (control group), G2 (treated with *P. djamor* cell-free extract), G3 (treated with 1% w/v papain), and G4 (treated with *P. djamor* cell-free extract + 1% w/v papain). This experiment was conducted in 200-µL microtubes. Each experimental group consisted of eight replicates. The experiment was carried out at two distinct time intervals: 2 hours and 24 hours (Silva et al., 2024a).

For the 2 hours assay, a suspension containing approximately 30 *Panagrellus* sp. individuals per 20 µL was used. For the 24 hours assay, a suspension with about 50 *Panagrellus* sp. individuals per 20 µL was employed. The following was added to each experimental group: G1—20 µL of the nematode suspension + 20 µL of distilled water; G2—20 µL of the nematode suspension + 20 µL of *P. djamor* cell-free extract; G3—20 µL of the nematode suspension + 20 µL of the 1% (w/v) papain solution; and G4—20 µL of the nematode suspension + 20 µL of the combined solution (1% w/v papain + *P. djamor* cell-free extract). The microtubes were transferred to an incubator and maintained at 28 ± 1 °C for the established time intervals. Intact nematodes were counted under a microscope using a 10× objective.

Protease stability assay

The stability of the proteolytic activity of the combination of *P. djamor* proteases and papain was evaluated at different time intervals: 0, 2, and 24 hours. Three experimental groups were established: G1 (*P. djamor* cell-free extract), G2 (1% w/v papain), and G3 (*P. djamor* cell-free extract + 1% w/v papain). All assays were performed in triplicate. In each group, the final volume was 2 mL. The microtubes were transferred to an incubator and maintained at 28 ± 1 °C for the established time intervals. The experimental conditions for determining proteolytic activity followed the same protocol described in the Proteolytic assay section.

Protease activity inhibition assay

The inhibition of the proteolytic activity of the combination of *P. djamor* proteases and papain was evaluated using PMSF. PMSF was purchased from Amresco and used at a final concentration of 5 mM (Adinarayana et al., 2003). One hundred microliters (100 µL) of each sample, G1 (*P. djamor* cell-free extract), G2 (1% w/v papain), and G3 (*P. djamor* cell-free extract + 1% w/v papain), were pre-incubated with 400 µL of buffer containing the inhibitor for 1 hour before substrate addition. The microtubes were transferred to an incubator and maintained at 28 ± 1 °C for the established time intervals. The experimental conditions for determining proteolytic activity followed the same protocol described in the Proteolytic assay section. Two experimental groups were established for each sample: a control group (without the inhibitor) and a treated group (with PMSF). All experiments were performed in triplicate.

Statistical analysis

Statistical analysis of the results was conducted using analysis of variance (ANOVA), considering significance levels of 1% and 5%. For mean comparisons, Tukey's test was applied, adopting the same probability levels (Ayres et al., 2003). Additionally, the mean percentage reduction in the number of *Panagrellus* sp. juveniles was calculated according to the following equation (Mendoza-De Gives & Vazquez-Prats, 1994):

$$\%\text{Reduction} = \frac{\overline{x}_{\text{control}} - \overline{x}_{\text{treatment}}}{\overline{x}_{\text{Control}}}$$

RESULTS AND DISCUSSION

Figure 1 presents the results of the nematicidal assay using *P. djamor* combined with papain at 2 hours (Figure 1A) and 24 hours (Figure 1B).

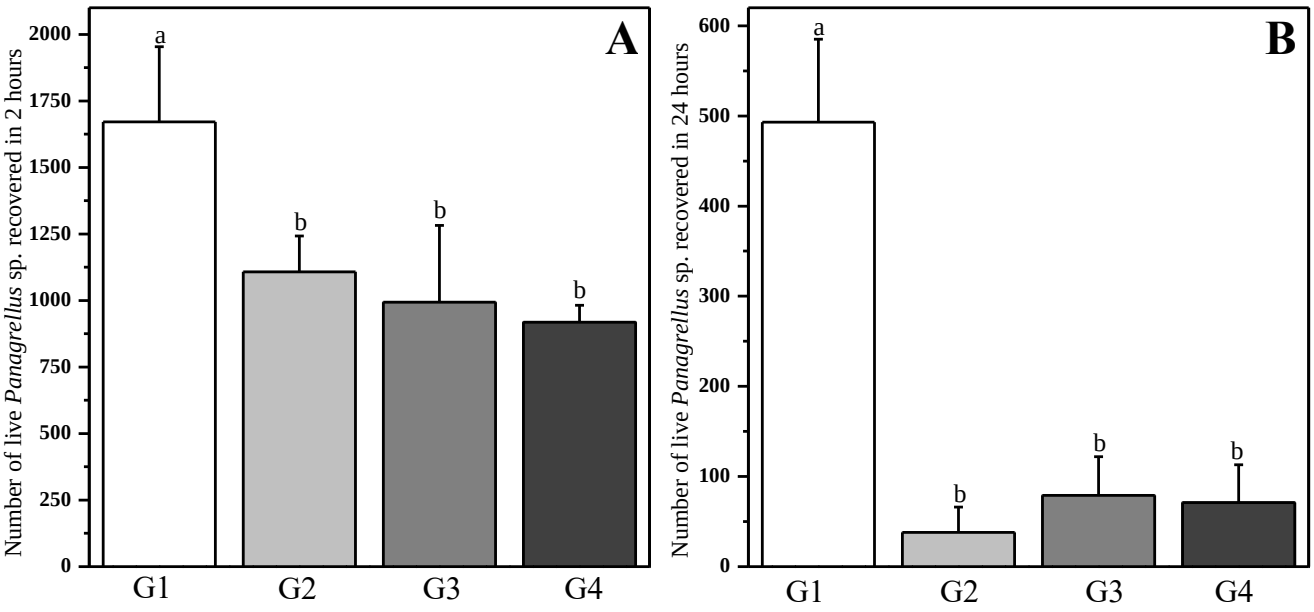

Figure 1. Evaluation of the combined nematophagous activity of *Pleurotus djamor* and the nematicidal activity of 1% (w/v) papain against Panagrellus sp. after 2 hours (A) and 24 hours (B). G1 (control group), G2 (treated with *P. djamor*), G3 (treated with 1% w/v papain), and G4 (treated with *P. djamor* + 1% w/v papain). Groups sharing the same letter do not differ significantly ($p > 0.05$). Groups with different letters exhibit a significant reduction ($p < 0.01$).

After 2 hours of exposure, all treated groups (G2, G3, and G4) showed a significant reduction in the number of *Panagrellus* sp. compared to the control group, G1 ($p < 0.01$). However, no significant differences were observed among the treatments themselves ($p > 0.05$). The percentage reductions relative to G1 were 34%, 41%, and 45% for G2, G3, and G4, respectively.

Consistently, the results obtained after 24 hours revealed the same pattern. All treated groups exhibited significant reductions compared to G1 ($p < 0.01$), while the differences among the treatments remained non-significant ($p > 0.05$). The reduction percentages were 86%, 84%, and 92% for G2, G3, and G4, respectively.

These results indicate that the combined use of the fungus *P. djamor* and papain did not result in an increased reduction percentage of *Panagrellus* sp. Nevertheless, it is noteworthy that the combination-treated group (G4) achieved a reduction statistically equivalent to that observed in the individually treated groups at both 2 hours and 24 hours ($p > 0.05$). This observation is important from a practical standpoint, asit indicates that combining the two does not compromise treatment efficacy, even in cases of potential mixing between the fungus and the enzyme under field conditions.

In line with this finding, Nyangwire et al. (2024) used the fungus *P. ostreatus* to control nematodes in eggplant roots and observed that, in addition to a reduction in the number of parasites, the treated plants exhibited better root growth. As a saprophytic fungus, *P. ostreatus* contributes to the decomposition of organic matter and, consequently, to greater nutrient availability in the soil. This improvement in plant nutrition enhances not only plant growth but also resistance to phytonematode infections.

Furthermore, spent mushroom substrate (SMS) represents a sustainable alternative to inorganic fertilizers due to its composition, which is rich in essential nutrients for plant growth (Rajavat et al., 2022; Idowu et al., 2023). Therefore, even in the absence of a synergistic effect between papain and *P. djamor*, the use of the mushroom remains promising: its combination with papain did not reduce its nematicidal potential, and its application can additionally contribute to soil fertilization (Yang et al., 2024).

Figure 2 presents the results of the nematicidal assay using the *P. djamor* cell-free crude extract combined with papain at 2 hours (Figure 2A) and 24 hours (Figure 2B). The proteolytic activity of the extract used in this assay was 43 ± 10 U/mL. After the 2-h assay, groups G3 and G4 showed a significant reduction in the number of *Panagrellus* sp. compared to G1 ($p < 0.01$), with no significant difference between them ($p > 0.05$). The reduction percentages were 38% and 29% for G3 and G4, respectively. Group G2 did not show a significant reduction relative to the control ($p > 0.05$), indicating that the extract alone was not effective within this time frame.

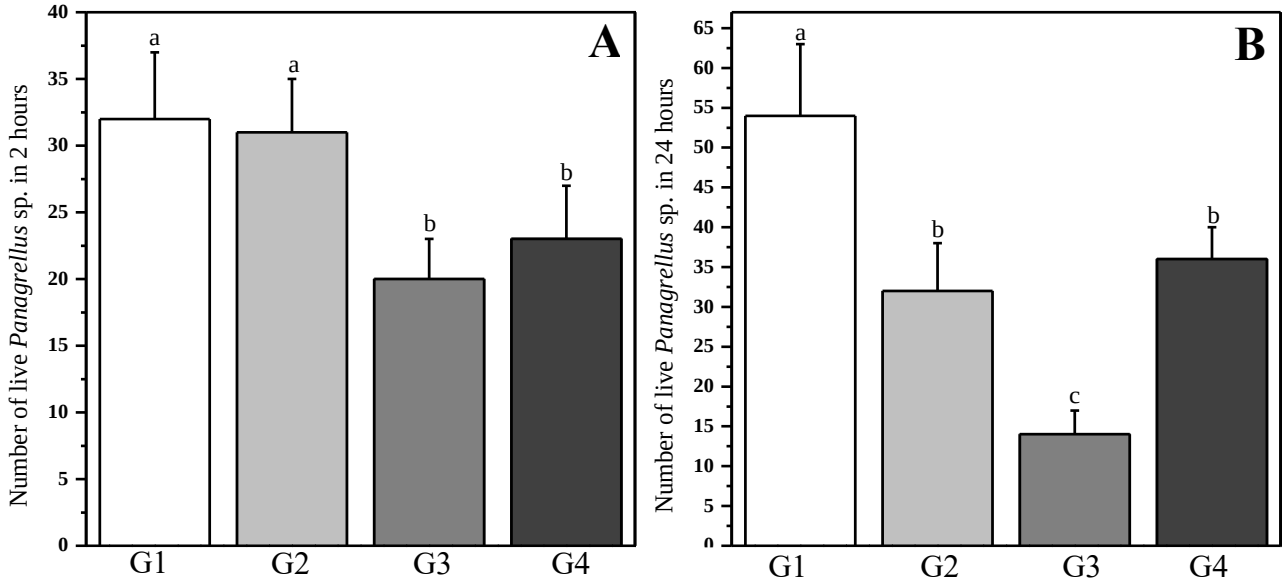

Figure 2. Evaluation of the combined nematicidal activity of *Pleurotus djamor* cell-free crude extract and 1% (w/v) papain against Panagrellus sp. after 2 hours (A) and 24 hours (B). G1 (control group), G2 (treated with *P. djamor* extract), G3 (treated with 1% w/v papain), and G4 (treated with *P. djamor* extract + 1% w/v papain). Groups sharing the same letter do not differ significantly ($p > 0.05$). Groups with different letters exhibit a significant reduction ($p < 0.01$).

At the 24 hours interval, all treatments showed a significant reduction compared to G1 ($p < 0.01$). Furthermore, group G3 exhibited a significant reduction compared to groups G2 and G4 ($p < 0.01$), demonstrating the superiority of papain alone at this exposure time. There was no significant difference in reduction between groups G2 and G4 ($p > 0.05$). The reduction percentages were 40%, 73%, and 32% for G2, G3, and G4, respectively.

The enzyme combination therefore exhibited distinct behaviors across the two evaluated intervals: at 2 hours, the reduction was statistically equivalent to that of papain alone; at 24 hours, the reduction was equivalent to that of the *P. djamor* extract alone. A comparison of the reduction percentages demonstrated that, at the 24 hours mark, papain significantly outperformed the other treatments ($p < 0.01$). This result is in agreement with the study by Castro et al. (2023), which demonstrated the promising effect of papain in the alternative and sustainable control of nematodes.

The nematicidal potential of papain against plant and animal parasitic nematodes is widely documented in the literature (Stepek et al., 2007; Soares et al., 2019; Pauta et al., 2024; Paula et al., 2025). The proteases present in papain are classified as cysteine proteases due to the presence of a cysteine residue in the catalytic site. This enzyme catalyzes the degradation of the nematode cuticle, a structure primarily composed of collagen, promoting its disruption

and consequently leading to the death of the organism (Silva-López et al., 2010; Njom et al., 2021).

Table 1 presents the enzymatic activities of the *P. djamor* cell-free extract (G1), 1% (w/v) papain (G2), and the combination of both (G3), evaluated at 0, 2, and 24 hours.

Table 1. Enzymatic activity (U/mL) of *Pleurotus djamor* cell-free crude extract (G1), 1% (w/v) papain (G2), and their combination (G3), evaluated at 0, 2, and 24 hours at 28 ± 1 °C in the stability assay.

	Enzymatic activity (U/mL)		
Interval	G1	G2	G3
0 hours	17 ± 1a	119 ± 1a	49 ± 7a
2 hours	16 ± 1a	109 ± 7a	29 ± 2b
24 hours	13 ± 1b	67 ± 3b	0 ± 0c

[a, b, c] Different letters within the same group indicate a significant decrease across the evaluated time intervals ($p < 0.05$). [a, a] Identical letters within the same group indicate no significant decrease across the evaluated time intervals ($p > 0.05$).

Group G1 exhibited activities of 17, 16, and 13 U/mL at the respective time points. No significant decrease was observed between 0 and 2 h ($p > 0.05$); however, the activity at 24 h was significantly lower ($p < 0.01$). For group G2, the activities were 119, 109, and 67 U/mL, showing a similar statistical behavior: no significant difference between the first two time points ($p > 0.05$) and a significant decrease after 24 h ($p < 0.01$).

In group G3 (combination), the observed activities were 49, 29, and 0 U/mL, exhibiting a significant decrease across all analyzed time points ($p < 0.01$). Notably, at all evaluated time points, the enzymatic activity of group G3 was lower than the sum of the activities of groups G1 and G2, and no proteolytic activity was detected at the 24-h mark.

This behavior suggests that one enzyme might be recognized as a substrate by the other, thereby explaining the progressive decrease in enzymatic activity when both are combined. This result aligns with López-Otín & Bond (2008), who pointed out that many proteases exhibit low substrate specificity and that some possess the ability to act on a broad spectrum of targets in a non-selective manner. Such a phenomenon represents a major limitation for the development of liquid formulations containing both proteolytic sources in direct contact.

Table 2 presents the relative percentages of enzymatic activities compared to the control group following the addition of the PMSF inhibitor. The observed residual activities were 0% for the *P. djamor* extract, 2% for papain, and 0% for their combination.

Table 2. Effect on the enzymatic activity of *Pleurotus djamor* cell-free crude extract, 1% (w/v) papain, and their combination, in the presence of the inhibitor: G1 (control, without inhibitor), G2 (PMSF – phenylmethylsulfonyl fluoride).

Group	% Relative Enzyme Activity		
	Pleurotus djamor extract	Papain 1% m/v	*P. djamor* extract + Papain 1% m/v
G1	100	100	100
G2	0	2	0

PMSF is a specific, irreversible inhibitor of serine proteases, although it can also inhibit some cysteine proteases, such as papain (Guo et al., 2016). The 98% inhibition of the protease activity in the papain solution confirms this sensitivity. Similarly, the complete inhibition of the proteases in the *P. djamor* extract suggests that these enzymes belong to the serine protease class, which are characterized by the presence of a serine residue in the catalytic site (Ma et al., 2024).

Considering that the inhibitor affected the activity of both proteases, the combination of the *P. djamor* extract and papain also had its enzymatic activity completely inhibited. This result corroborates the stability data and reinforces the classification of the fungal proteases as serine proteases, complementing evidence obtained in previous studies using zymography, in which at least two proteases with molecular weights between 75 and 100 kDa were identified (Silva et al., 2025).

Conclusion

The combination of *P. djamor* (or its proteolytic extract) and papain does not enhance the nematicidal activity against *Panagrellus* sp., refuting the hypothesis of a synergistic or additive effect between these proteolytic sources. However, the association also does not compromise the individual efficacy of either component, indicating the possibility of using both control methods in an integrated manner if combining different management strategies is required.

The PMSF inhibition profile indicated that *P. djamor* proteases belong to the serine protease class. Papain, despite being a cysteine protease, also exhibited sensitivity to the inhibitor, resulting in the total inhibition of proteolytic activity when both were combined.

In the field of applied biotechnology, this study reinforces the need to evaluate the biochemical compatibility between biopesticide components before assuming additive or synergistic effects. This precaution is particularly relevant when proteases with different catalytic specificities are involved, as the combination of these enzymes resulted in a loss of activity over time, possibly due to cross-degradation, wherein one protease acts as a substrate

for the other. Such interactions must be carefully considered in the development of formulations intended for biological control.

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Author contributions

Silva Adriane: Conceptualization, Methodology, Investigation, Formal analysis, Writing – Original draft preparation, Writing – Review and Editing. Silva Ana: Investigation, Writing – Review and Editing. Alves Amanda: Investigation, Writing – Review and Editing. Sales Nivia: Investigation, Writing – Review and Editing. Dias Eustáquio: Resources, Writing – Review and Editing. Soares Filippe: Conceptualization, Methodology, Supervision, Writing – Original draft preparation, Writing – Review and Editing.

Declaration of competing interest

The authors declare no known competing financial interests or personal relationships that influenced the results reported in this paper.

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9 CONSIDERAÇÕES FINAIS

As pesquisas densenvolvidas nesta tese demonstrou que o uso de fungos do gênero *Pleurotus* e de suas proteases constitui uma alternativa biotecnológica e sustentável para o controle de patógenos na agropecuária. A pesquisa confirmou que diferentes espécies do gênero, com destaque para o *P. djamor*, possuem alta eficácia nematicida, fundamentada em um mecanismo de ação que integra a produção de toxinas paralisantes e a secreção de proteases responsáveis pela catálise de degradação da cutícula ou da casca dos ovos dos parasitas.

No âmbito da produção enzimática, a Fermentação em Estado Sólido utilizando o farelo de trigo revelou-se superior à fermentação submersa, alcançando atividades proteolíticas até cinco vezes maiores. A otimização do meio de cultura identificou que a umidade é a variável que influencia o fungo a produzir as proteases, permitindo a valorização de resíduos agroindustriais e alinhando o processo aos princípios da economia circular.

A versatilidade das proteases de *P. djamor* foi evidenciada pela sua capacidade de controlar um amplo espectro de alvos, incluindo: Parasitas gastrointestinais de ruminantes, com reduções significativas de larvas de *Haemonchus* spp. e *Trichostrongylus* spp. mesmo em coproculturas. Fitonematoides, como o *M. incognita*, onde o extrato bruto livre de células reduziu significativamente a formação de galhas e a quantidade de ovos em tomateiros sem apresentar toxicidade para as plantas.

Além disso, as proteases apresentaram bons resultados no controle de cestódeos de importância na pecuária, demonstrando ação ovicida contra *Moniezia* sp. e também sobre os ovos de *T. solium* que é um parasita zoonótico.

A caracterização bioquímica por meio do zimograma revelou a presença de pelo menos duas serino proteases principais (com massas entre 75 e 100 kDa), cuja atividade é sensível ao inibidor PMSF. Um resultado inédito e fundamental para o desenvolvimento de futuros bioinsumos foi a constatação de que a associação entre o extrato *de P. djamor* e a papaína não

gera sinergismo, devido à possível degradação proteolítica cruzada entre as enzimas, o que limita a eficácia de longo prazo de formulações combinadas.

Em suma, esta tese contribui para o conceito de Saúde Única ao propor estratégias que minimizam o uso de agroquímicos e reduzem a resistência parasitária. Como perspectivas, recomenda-se o aprofundamento em técnicas de imobilização enzimática dessas proteases, consolidando-as como alternativas sustentáveis, competitivas e ecológicas no mercado global de defensivos agrícolas e veterinário.