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# Analysis of the effect of *Bacillus velezensis* culture filtrate on the growth and proteome of *Cladobotryum mycophilum*

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ABSTRACT

*Cladobotryum mycophilum*, the causative agent of cobweb disease on *Agaricus bisporus* results in significant crop losses for mushroom growers worldwide. Cobweb disease is treated through strict hygiene control methods and the application of chemical fungicides but an increase in fungicide resistant *Cladobotryum* strains has resulted in a need to develop alternative biocontrol treatment methods. The aim of the work presented here was to evaluate the response *of C. mycophilum* to *a Bacillus velezensis* isolate to assess its potential as a novel biocontrol agent. Exposure of 48 hr C. *mycophilum* cultures to 25% v/v 96 hr *B. velezensis* culture filtrate resulted in a 57% reduction in biomass ($P < 0.0002$), a disruption in hyphal structure and morphology, and the appearance of aurofusarin, a secondary metabolite which is a known indicator of oxidative stress, in culture medium. Proteomic analysis of *B. velezensis* culture filtrate revealed the presence of peptidase 8 (subtilisin), peptide deformylase and probable cytosol aminopeptidase which are known to induce catalytic activity. Characterisation of the proteomic response of *C. mycophilum* following exposure to *B. velezensis* culture filtrate revealed an increase in the abundance of a variety of proteins associated with stress response (ISWI chromatin-remodelling complex ATPase ISW2 (+24 fold), carboxypeptidase Y precursor (+3 fold) and calmodulin (+2 fold). There was also a decrease in the abundance of proteins associated with transcription (40 S ribosomal protein S30 (−26 fold), 40 S ribosomal protein S21 (−3 fold) and carbohydrate metabolism (L-xylulose reductase (−10 fold). The results presented here indicate that *B. velezensis* culture filtrate is capable of inhibiting the growth of *C. mycophilum* and inducing a stress response, thus indicating its potential to control this important pathogen of mushrooms.
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## 1. Introduction

Mushrooms are susceptible to a wide range of viral, bacterial and fungal pathogens that adversely affect quality and reduce yield. One of the most challenging fungal pathogens of mushrooms, *Cladobotryum mycophilum*, is difficult to control and the recent appearance of fungicide-resistant isolates has compromised treatment of crops (Grogan and Gaze 2000; Grogan 2008). There is an urgent need to identify novel ways to control this increasingly serious pathogen and biocontrol agents may represent a new and effective way to control infection. Other fungal pathogens that affect mushroom cultivation such as wet bubble disease (*Mycogyne perniciosa*), dry bubble disease (*Lecanicillium fungicola*) and green mould disease (*Trichoderma aggressivum*) have also developed resistance to some fungicides and may require biocontrol agents for control in the near future (Fletcher and Gaze, 2008).

Cobweb disease due to *C. mycophilum* can result in significant crop losses, product deformation and consequential revenue loss for mushroom farmers. Although cobweb disease is known to infect various economically important mushroom species (*Pleurotus eryngii*, *Flammulina velutipes*, and *Ganoderma lucidum*) it is mostly associated with *Agaricus bisporus* (Lange) [Imbach] (Back et al., 2010; Gea et al., 2011; Kim et al., 2012; Zuo et al., 2016; Gea et al., 2017). *A. bisporus* is an important edible mushroom species, accounting for around 15% of cultivated mushroom production worldwide (Royse et al., 2017). The widespread use of white hybrid strains derived from HorstU1 in *A. bisporus* cultivation means that world production is effectively a monoculture, and this has made the species more universally susceptible to attack by the same

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**List of abbreviations**

| | |
|---|---|
| MBC | methylbenzimidazole carbamate |
| IPM | integrated pest management |
| SUD | Sustainable Use of Pesticides Directive |
| GO | gene ontology |
| BP | biological process |
| MF | molecular function |
| CC | cellular component |
| SSDA | statistically significant differentially abundant |
| LFQ | Label free quantitative-proteomic |
| MEA | malt extract agar |
| NB | nutrient broth |
| NA | nutrient agar |
| SDB | Sabouraud dextrose liquid broth |
| PDA | potato dextrose agar |
| CF | culture filtrate |
| ANOVA | analysis of variance |
| PCA | principal component analysis |
| GRAS | generally regarded as safe |

pathogens due to its lack of genetic diversity (Sonnenberg et al., 2017).

Cobweb disease is caused by several members of the *Cladobotryum* genus including *Cladobotryum dendroides, C. mycophilum, Cladobotryum varium, Cladobotryum multiseptatum, Cladobotryum asterophorum, Cladobotryum semicirculare and Cladobotryum protrusum*, occurring naturally in the wild on several different fungal basidiomycete taxa including polypores (Tamm and Põldmaa 2013). Historically, *C. dendroides* (teleomorph: *Hypomyces rosellus*) (Gams and Hoozemans 1970) was most associated with cobweb incidence in commercial *A. bisporus* production. Today it is believed that *C. mycophilum* (teleomorph: *Hypomyces odoratus*) (Gams and Hoozemans 1970), has overtaken *C. dendroides* as the most common causative agent of cobweb disease (McKay et al., 1999; Tamm and Põldmaa 2013). A strain of *Cladobotryum*, resistant to methylbenzimidazole carbamate (MBC) fungicides which had similar characteristics to *C. mycophilum* but lacked the camphor odour distinctive of the species was classified as *C. mycophilum* type II (McKay et al., 1999).

In *A. bisporus* cultivation *Cladobotryum* species grow over the casing layer with a white, fluffy mass of mycelium which can spread to, and envelope neighbouring fruit bodies. If left untreated, the fruit body will usually present first with discolouration and eventually rot and become unsellable. If not dealt with immediately, localized outbreaks will appear throughout the mushroom crop. *C. dendroides* spores are very easily dispersed through air conditioning systems or through the action of watering/salting (Adie and Grogan 2000; Adie et al., 2006). Conidial dispersion results in the appearance of brown, irregular spots on the mushrooms when the spores land on the *A. bisporus* caps and germinate (Adie and Grogan 2000). The infection then spreads to adjacent mushrooms on the bed, eventually resulting in rotted crops, which are unfit for sale. Conidial dispersion also results in the establishment of secondary points of infections which further reduces crop yield (Adie et al., 2006).

Cobweb disease is controlled on mushroom farms through strict hygiene control methods and fungicide application. It is important to treat cobweb disease as soon as it becomes apparent on the mushroom farm to prevent conidial dispersion from getting out of control. The recommended treatment method for cobweb disease is to cover the diseased area with a thick layer of damp paper and generously cover with salt (Grogan and Gaze 2008). This is done to prevent the dispersion of conidia from the infected area, however caution is needed as salting without damp paper has been shown to aid conidial dispersion (Adie et al., 2006).

Currently, there are only a limited number of chemical fungicides approved for use on mushroom crops. Prochloraz is mostly used in Europe, while chlorothalonil and thiabendazole are popular in North America (Grogan, 2008; Potocnik et al., 2015). Reliance upon a select number of fungicides has resulted in various mushroom pathogens becoming resistant to current treatment options (Grogan, 2008). In the 1970s, MBC fungicides such as benomyl and carbendazim were commonly applied to mushroom crops to protect against cobweb disease (Fletcher, 1973). Resistance to these fungicides emerged within a decade, and were first identified in Great Britain (Gaze, 1995; McKay et al., 1998). These resistant strains are also present in Irish isolates, and were responsible for a crisis which reached epidemic proportions in the 1990s (Adie et al., 2006). A new fungicide, metrafenone, was approved for use against cobweb disease in several European countries in 2017 (Carrasco et al., 2017; Pyck et al., 2016) but there are already widespread reports that metrafenone-resistance has developed. Most countries are now planning to reduce the use of fungicides and implement integrated pest management (IPM) strategies. This strategy is promoted under the Sustainable Use of Pesticides Directive (SUD) 2009/128/EC (Anon, 2009). When available, non-chemical biocontrol treatments should be used in place of chemical fungicides, which can be harmful to both human health and the environment.

The application of biocontrol agents offers a potential alternative to fungicide use in mushroom crop cultivation. *Bacillus* species are commonly studied as potential biocontrol agents due to their ability to out compete fungal pathogens in spatial and nutritional competition (Borriss, 2015; Pandin et al., 2017). Their use is complicated by the fact that both host, and pathogen are fungal species. SERENADE® (AgraQuest Inc.) is a commercially available biocontrol agent which has been used to treat *T. aggressivum* and *L. fungicola* successfully (Stanojević et al., 2019). Serenade is diluted into water and sprayed onto crops prior to the formation of mushroom pins. It uses *Bacillus velezensis* QST 713 as its active ingredient (Pandin et al., 2018). It has not been shown to be effective against cobweb species. Kosanovic et al. (2021) isolated an environmental *B. velezensis* strain (strain Kos) which was able to control the growth of *T. aggressivum* without adversely affecting *Agaricus* growth. Here, we aim to investigate the potential of this *B. velezensis* strain as a biocontrol agent against *C. mycophilum in-vitro*.

## 2. Materials and methods

### 2.1. Culture conditions

A carbendazim and metrafenone resistant *C. mycophilum* type II strain (isolate 1546), isolated from an infected mushroom crop and stored in the Teagasc Ashtown culture collection (Dublin, Ireland), was used in these studies. Cultures were grown on malt extract agar medium (MEA) (Oxoid) at 25 °C for up to 3 days, in the dark. Liquid cultures of *C. mycophilum* were grown in Sabouraud dextrose liquid broth (SDB), at 25 °C, 120 rpm for 48 h.

*B. velezensis* (strain Kos) (Kosanovic et al., 2021) was obtained from liquid nitrogen stocks at Maynooth University (Kildare, Ireland). *B. velezensis* cultures were maintained on nutrient agar (NA) (Oxoid) and were grown at 25 °C, in the dark for 2–3 days.

### 2.2. *B. velezensis* culture filtrate (CF) collection and separation

*B. velezensis* culture filtrate (CF) was established by adding a loopful of *B. velezensis* from an NA plate culture, into nutrient broth (NB) (Oxoid) (50 mls). Flasks were incubated at 30 °C at 120 rpm in an orbital incubator. At 24 h, 48 h, 72 h and 96 h, CF was collected by centrifugation (20 min, x 5000 g), and was passed through a 0.45 μm filtropur S filters (Sarstedt Ltd). CF was stored at −20 °C until needed.

The 96 h CF was filtered through a 0.2 μm filtropur S filters (Sarstedt Ltd) and separated into four fractions; >3 kda polar, > 3kda non-polar, <3 kda polar and <3 kda non-polar using Vivaspin 20 centrifugal concentrator (sartorius) and C18 cartridges (Sep-Pak ® Vac 3 cc 200 mg). Samples were lyophilised and resuspended in dd$H_2O$.

### 2.3. The effect of *B. velezensis* culture filtrates on the growth of *C. mycophilum* to identify inhibitory component

A *C. mycophilum* conidial suspension was prepared and its conidia concentration was determined using a haemocytometer. The suspension was then adjusted to x$10^5$/ml. A 100 μl aliquot (x$10^4$ conidia/plate) was spread onto potato dextrose agar (PDA) plates using a sterile spreader, and plates were left to dry for 15 min. Wells (8 mm, diameter) were added to the PDA plates and 50 μl of the four 96 h B. *velezensis* culture filtrate fractions were applied to the wells. Plates were incubated at 25 °C, in the dark for three days.

### 2.4. Label free qualitative proteomics of polar, >3 kda fraction from *B. velezensis* 96 h culture filtrate

The plate inhibition assay identified the >3 kda, polar sample as inhibitory against *C. mycophilum*. This sample was acetone precipitated overnight. The protocol for protein extraction and mass spectrometry preparation was as described in Margalit et al. (2020).

Samples were run on a QExactive (ThermoFisher Scientific, USA) high resolution accurate mass spectrometer connected to a Dionex Ultimate 3000 (RSLCnano) chromatography system. Peptides were separated by an increasing acetonitrile gradient from 2% to 40% on a Biobasic C18 Picofrit column (100 mm length, 75 mm ID), using a 120-min reverse phase gradient at a low rate of 250 nl/min. A full MS scan of range 200–2000 was followed to select the 15 most intense ions prior to MS/MS.

Qualitative analysis was carried out using Proteome Discoverer 1.4 and Sequest HT (SEQUEST HT algorithm, Thermo Scientific). Proteins which were identified within the >3 kda *B. velezensis* CF sample were queried against the *B. velezensis* proteome (identifier 492670) (Genome assembly accession: CP026610, proteome ID: UP000425588) downloaded from www.uniprot.org. Proteins with a score of <0 and a peptide number <2 were excluded from the analysis.

### 2.5. The effect of *B. velezensis* culture filtrate on the growth of *C. mycophilum* in-vitro

*C. mycophilum* (x$10^4$ conidia/plate) was spread onto PDA plates using a sterile spreader and was left to dry for 15 min. Once dry, wells (8 mm diameter) were cut in the PDA plates and 50 μl of *B. velezensis* culture filtrate at various time points (24–96 h) were added into the wells. *B. velezensis* culture drops (10 μl) were also added directly onto plates. Samples of both CF and cell culture drops were applied in triplicate. All plates were incubated at 25 °C for three days. NB was used as a negative control. In order to determine the optimal timepoint of *B. velezensis* CF, samples from 24, 48, 72, 96, 120, 144, 168 and 196 h were applied to PDA plates which contained *C. mycophilum* (x$10^4$ condida/plate) as described above. Zones of inhibition were calculated by measuring the distance (mm) from the centre of the well to the furthest point of growth disturbance. This radius value was then used to calculate the area of the zone ($A = \pi r^2$).

Cultures of *C. mycophilum* (x$10^3$ conidia/ml) were grown in 50 mls of SDB for 48 h at 25 °C and 120 rpm. Cultures were then supplemented with 96 h B. *velezensis* culture filtrate at a concentration of 25% v/v for treatment 1 and 12% v/v for treatment 2. Control flasks were supplemented with NB to a 25% v/v concentration. Cultures were incubated under the same conditions for a further 24 h. This experiment was carried out with five replicates per treatment. The mycelium wet weight was measured once the culture filtrate had been removed.

One protein present within the *B. velezensis* 96 h CF which was of particular interest was peptidase S8 or subtilisin. Flasks of *C. mycophilum* (x$10^3$) (30 mls) were grown for 48 h at 25 °C and 120 rpm. The flask were then supplemented with 12.5% v/v purified subtilisin dissolved in PBS (1 mg/ml) (Sigma–Aldrich). Flasks were returned to the same conditions for a further 24 h. Control flasks were supplemented with 12.5% v/v PBS. Each treatment was done in triplicate. After 24 h, the mycelium within the flasks were weighed and the effect of subtilisin on mycelium wet weight per treatment was determined.

### 2.6. Fluorescent microscopy

Stationary phase *C. mycophilum* (x$10^3$ conidia/ml) was grown for 48 h and supplemented with either 24 or 96 h B. *velezensis* culture filtrate (25% v/v) for a further 24 h. Fungal cultures supplemented with 25% v/v nutrient broth were used as a control. Hyphae were collected from the cultures and a small sample from each treatment was applied to a glass slide. Hyphae were first washed with PBS (50 μl) three times. Calcofluor white (25 μl, Sigma–Aldrich) was then applied to the hyphae for 5 min at room temperature. The excess stain was washed off with PBS. A cover slip was placed directly on top of the stained hyphae. Slides were visualised on Olympus BX51 fluorescent microscope (X40 lens).

### 2.7. Label free quantitative proteomics of *C. mycophilum* treated with *B. velezensis* culture filtrates

Proteins were extracted from *C. mycophilum* mycelium which was grown for 48 h before it was supplemented either with 12.5 ml/50 ml B. *velezensis* 96 h CF (treatment 1), 6.25 ml/50 ml B. *velezensis* 96 h CF (treatment 2), or 12.5 ml/50 ml NB (control). Each treatment was performed in replicates of five. The protocol for protein extractions and mass spectrometry run are described in Margalit et al. (2020). For the quantitative run, 0.75 μg of the resuspended *C. mycophilum* digested protein mix was applied to the QExactive rather than 0.50 μg. The steps for proteomic data analysis are described in Margalit et al. (2020).

Quantitative analysis was preformed using Andromeda search engine in Max-Quant (version 1.6.17 https://www.maxquant.org/). Max-Quant was used to identify the proteins within the sample and to correlate them against the *Trichoderma harzianum* proteome (strain CBS 226.95 (Genome assembly accession: #MBGI01000000, proteome ID: UP000241690) downloaded from www.uniprot.org. There is no *Cladobotryum* database currently available on UniProt, hence why a closely related species, *T. harzianum* was chosen (Xu et al., 2020).

Perseus (version 1.6.14.0) was employed for data and statistical analysis as well as graphics visualisation (Margalit et al., 2020). A principal component analysis (PCA) was generated with the

resulting dataset. Gene ontology (GO) mapping was performed in Perseus, the UniProt gene IDs for all identified proteins were quired against a *T. harzianum* annotation file generated from Blast 2 Go tool (https://www.blast2go.com/) and uploaded to Perseus. This provided terms for gene ontology biological process (BP), gene ontology cellular component (CC), gene ontology molecular function (MF) and UniProt name for each protein. ANOVA of significance and t-tests between the treated groups was performed using a p-value of 0.05. Volcano plots were generated by plotting the $\log_2$ fold change on the x axis against the log p values on the y axis for each pairwise comparison. Statistically significant and differentially abundant (SSDA) proteins (ANOVA, $p < 0.05$) with a relative fold change greater than $\pm$ 0.58 were retained for analysis. SSDA proteins were Z-score normalised and then used for hierarchical clustering to produce a heat map. The mass spectrometry proteomics data have been deposited to the ProteomeXchange Consortium via the PRIDE partner repository with the dataset identifier PXD026467.

## 3. Results

### 3.1. Analysis of the effect of B. velezensis culture filtrate on the growth of C. mycophilum

The effect of *B. velezensis* CF on *C. mycophilum* growth was determined as described and the greatest zone of inhibition was evident when 96 h CF was used (Fig. 1). Clear zones of inhibition could be found surrounding areas where *B. velezensis* CF had been applied on PDA plates containing *C. mycophilum* (x$10^4$/plate) (Fig S1A). CF samples from all collection time points (24, 48, 72 and 96 h) produced distinct zones of clearance. Bacterial cells from each time point also significantly inhibited the growth of *C. mycophilum* (x$10^4$/plate) on PDA plates (Fig. S1B).

The growth within *C. mycophilum* flask cultures which were supplemented with *B. velezensis* 96 h CF was assessed after 24 h. The presence of *B. velezensis* culture filtrate reduced the growth of *C. mycophilum* hyphae within these flask cultures. The average wet weight in control flasks, which contained *C. mycophilum* (x$10^3$/flask) and NB was 2.8±0.46 g (grams). In flasks which contained either 25% v/v or 12.5% v/v, *B. velezensis* 96 h CF, the growth of *C. mycophilum* was reduced by 57% ($p < 0.0002$). The average wet weight was 1.2 $\pm$ 0.24 g and 1.2 $\pm$ 0.18 g for treatment 1 and treatment 2 respectively (Fig. 2). There was also a physical difference between control and treatment flasks as the medium in the treated flasks was reddish/brown colour (Fig. S2).

### 3.2. Fluorescent microscopic examination of C. mycophilum cultures exposed to B. velezensis culture filtrate

The images produced of the *C. mycophilum* hyphae by fluorescent microscopy show that both hyphal growth and development were seriously disrupted when exposed to *B. velezensis* CF. Hyphae taken from control were well defined and linear (Fig. 3A). Hyphae of *C. mycophilum* treated either with 24 h or 96 h *B. velezensis* CF were clearly disrupted and contained globular structures (Fig. 3B and C). Fewer hyphae were observed in these treatments, and when present, hyphae appeared short and rounded. The internal organisation of the hyphae also seemed to be adversely affected by *B. velezensis* CF.

### 3.3. The effect of B. velezensis culture filtrate fractions on the growth of C. mycophilum to identify inhibitory component

*B. velezensis* CF was fractionated to assist in identifying the agents in it that might be responsible for growth inhibition and distortion to hyphae. A clear zone of inhibition was visualised surrounding the area on the plate where the >3kda, polar samples had been applied. A zone of clearance was also identified around the >3kda, non-polar sample. No inhibition could be seen around the <3kda polar and non-polar samples (Fig. S3). This would suggest that the component within the CF which causes inhibition is > 3 kda. As the zone of inhibition was strongest around the >3 kda, polar sample, this was chosen to proceed with for protein extraction and mass spectrometry.

### 3.4. Label free qualitative proteomics of B. velezensis >3 kda, polar 96 h culture filtrate

Qualitative proteomic analysis identified a number of proteins which were present within the *B. velezensis* CF > 3kda, polar sample that may be causing inhibition of *C. mycophilum* growth. The majority of proteins identified within this sample were peptidases (peptidase S8 (subtilisin), aminopeptidase YsdC, probable cytosol aminopeptidase) (Table S1). There was also a number of other

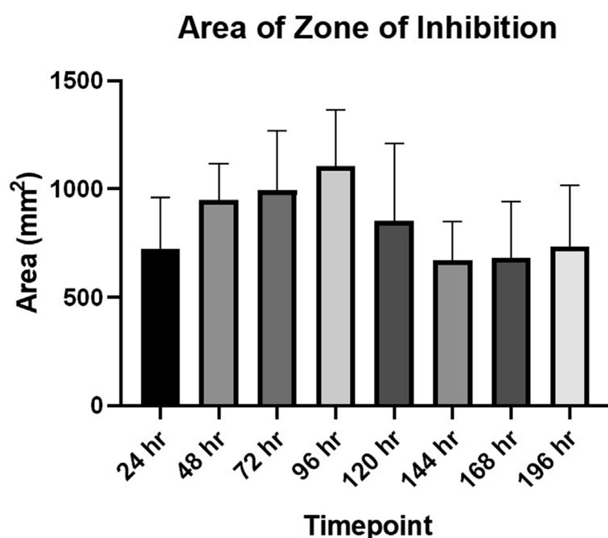


**Fig. 1.** The average area of the zone of inhibition produced by *B. velezensis* CF at various timepoints against *C. mycophilum* (x$10^4$).

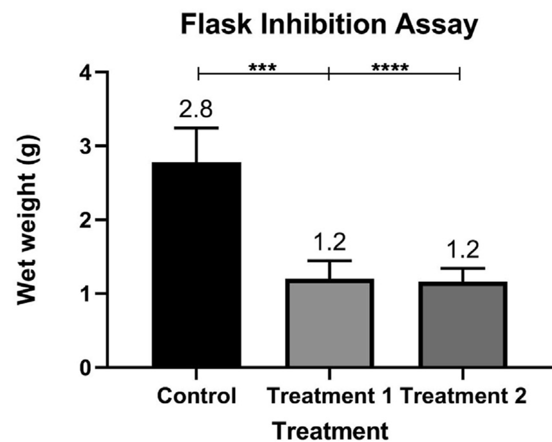


**Fig. 2.** Flasks of *Cladobotryum* (x$10^3$) were grown for 48 h and then supplemented with either NB (control), 25% v/v 96 h CF (treatment 1) or 12.5% v/v 96 h CF (treatment 2) for 24 h. Average hyphae wet weight per treatment is graphed above. Error bars represent standard deviation. ***: P value 0.0002, ****: P value < 0.0001.

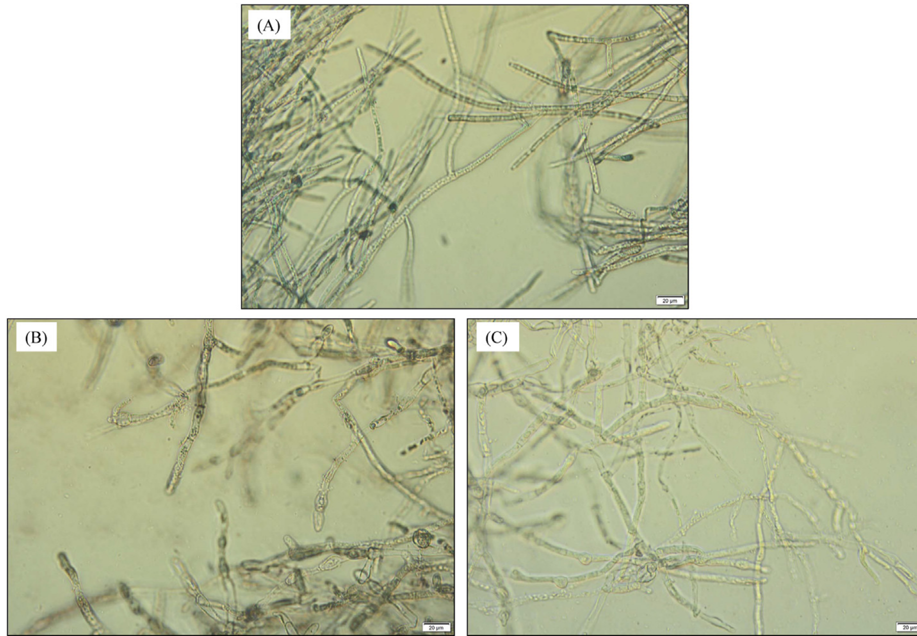


**Fig. 3.** Hyphae from treatment and control samples were visualised using an Olympus microscope at magnification X20. Control hyphae (A) appeared to be healthy and well-structured and defined. *Cladobotryum* treated with 24 h CF (B) and 96 h CF (C) appeared to be damaged and twisted.

enzymes present including; catalase, citrate synthase, sucrose-6-phosphate hydrolase and peptide deformylase.

The effect of purified subtilisin on *C. mycophilum* growth was assessed after 24 h. The average wet weight of *C. mycophilum* exposed to 12.5% v/v subtilisin was 0.49±0.2 g. This represents a percentage decrease of 28% compared to control flasks, which had an average wet weight of 0.68±0.2 g (Fig S4).

### 3.5. *Label free quantitative proteomics of* C. mycophilum *treated with* B. velezensis *culture filtrates*

The whole cell proteomic response of *C. mycophilum* when exposed to *B. velezensis* 96 h culture filtrate (12.5% & 25% v/v) was investigated using label free quantitative (LFQ) proteomics. In total, 1279 proteins were initially identified and 733 remained after various filtration processes which removed proteins only identified by site and potential contaminants. According to analysis carried out in Perseus, there were 81 statistical SSDA proteins in treatment 1 (41 increased and 40 decreased) (analysis of variance [ANOVA], $P < 0.05$). In treatment 2, there were 41 SSDAs (24 increased and 17 decreased) ([ANOVA], $P < 0.05$). Each of these SSDA proteins had a fold change value of $> \pm 0.58$. All of these proteins were included in further statistical analysis. A total of 26 SSDAs were common to both treatment 1 and treatment 2. Treatment 1 therefore had 55 exclusive SSDAs, while treatment 2 had 15. The fold change of these common SSDAs appear to be higher in treatment 1 compared to treatment 2, meaning that the mutual SSDAs were found at a greater extent in treatment 1. The higher concentration of *B. velezensis* CF in treatment 1 (25% v/v) may be responsible for this larger difference in SSDA's compared to treatment 2 (12.5%v/v).

A PCA performed on filtered proteins confirmed that the two sets of treatment samples, were significantly different from the control (Fig. 4A). The control samples clustered on their own, while treatment samples clustered close together but remained separate from one another. This indicates a clear difference between proteomes of treated samples and control samples.

Hierarchal clustering performed in Perseus, shows that there is a clear difference in protein abundance within the control and treatment sample. This difference is further highlighted in a heat map (Fig. 4B) which was generated from data gathered during Perseus analysis. In areas where there is decreased relative protein abundance in the treatments, there is increased relative protein abundance compared to the control. This further proved that the presence of the *B. velezensis* CF is influencing the growth of *C. mycophilum.*

Volcano plots (Fig. 5) show the distribution of SSDA proteins. Proteins which increased in relative abundance in treatment 1 (*C. mycophilum* treated with 25% v/v *B. velezensis* CF) compared to the control include; ISWI chromatin-remodelling complex ATPase (24-fold), AAA ATPase (8-fold), t-snare syntaxin (5-fold) and carboxypeptidase Y precursor (3-fold). Proteins which decreased in treatment 1 compared to the control include; 40 S ribosomal protein S30 (−26-fold), ATP synthase d chain (−12-fold) and L-xylulose b5 reductase 2 (−10-fold) (Fig. 5A).

Proteins which increased in relative abundance in treatment 2 (*C. mycophilum* treated with 12% v/v *B. velezensis* CF) compared to the control include; Prenylated Rab acceptor 1 (9-fold), GTP-binding protein rho2 (4-fold) as well as ISWI chromatin-remodelling complex ATPase (21-fold) and AAA ATPase (6-fold), which were also upregulated in treatment 1. Proteins which decreased in treatment 2 included porphobilinogen deaminase (−19-fold), mitochondrial inner membrane translocase subunit TIM44 (-5-fold) as well as 40 S ribosomal protein S30 -(16-fold) and L-xylulose reductase (-5-fold) which were also decreased in treatment 1 (Fig. 5B). The most abundant proteins, either up or down-regulated in either group, are listed (Table S2-5).

GO mapping from the Blast2Go software tool was also carried out on SSDA proteins from each treatment group. Several GO terms which belong to biological process (BP) were enriched for treatments 1/2 compared to the control including; Proteolysis involved in cellular protein catabolism, cellular catabolic process and DNA repair. BP terms; translation, ribosome biogenesis and nucleotide metabolic process were enriched in control and down in

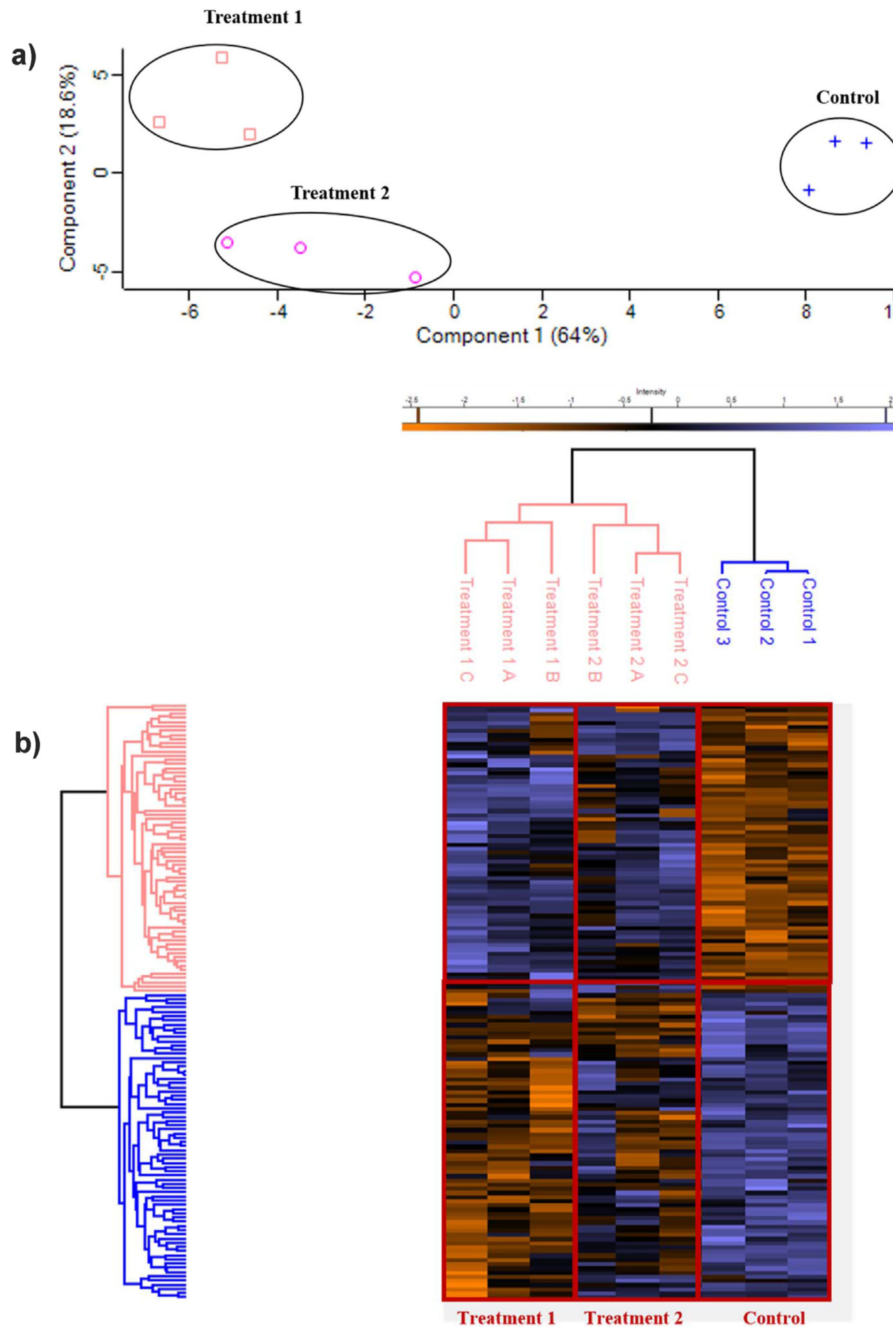


**Fig. 4. A.** Principal component analysis generated during Perseus analysis. The PCA groups control samples (+) together and away from treatment 1 (□) and 2 (o) samples. **B.** Heat map depicts the overall pattern of increased (purple) and decreased (orange) protein abundance within the samples Hierarchal clustering is also depicted and groups control samples and treatment samples on separate lineages.

treatments. Molecular functions (MF) such as ATP binding and magnesium ion binding were also higher in treatments compared to the control (Fig. S5).

## 4. Discussion

The results presented here indicate that the CF of a *B. velezensis* species has a negative effect on the growth of *C. mycophilum*. Both *B. velezensis* culture filtrate and bacterial cells were able to inhibit the growth of *C. mycophilum* on PDA plates. Flask assays showed that the presence of *B. velezensis* culture filtrate in *C. mycophilum* liquid culture, could reduce biomass accumulation by an average of 57%. A review of literature on *Cladobotryum* species suggest the red colour present in *C. mycophilum* cultures exposed to *B. velezensis* culture filtrate was due to the release of aurofusarin (Põldmaa 2011). This secondary metabolite is released by some *Cladobotryum* species, including *C. mycophilum* around 25 days into their growth. The red pigment is indicative of a cessation in growth and an oxidative stress response (Põldmaa, 2011; Cambaza, 2018). This is another indication that *B. velezensis* CF can halt the growth and development of *C. mycophilum.* Images of hyphae exposed to *B. velezensis* CF also confirm that the growth of *C. mycophilum* cells are stunted in the presence of *B. velezensis* CF. Treated hyphae are clearly being distorted while control hyphae appear healthy.

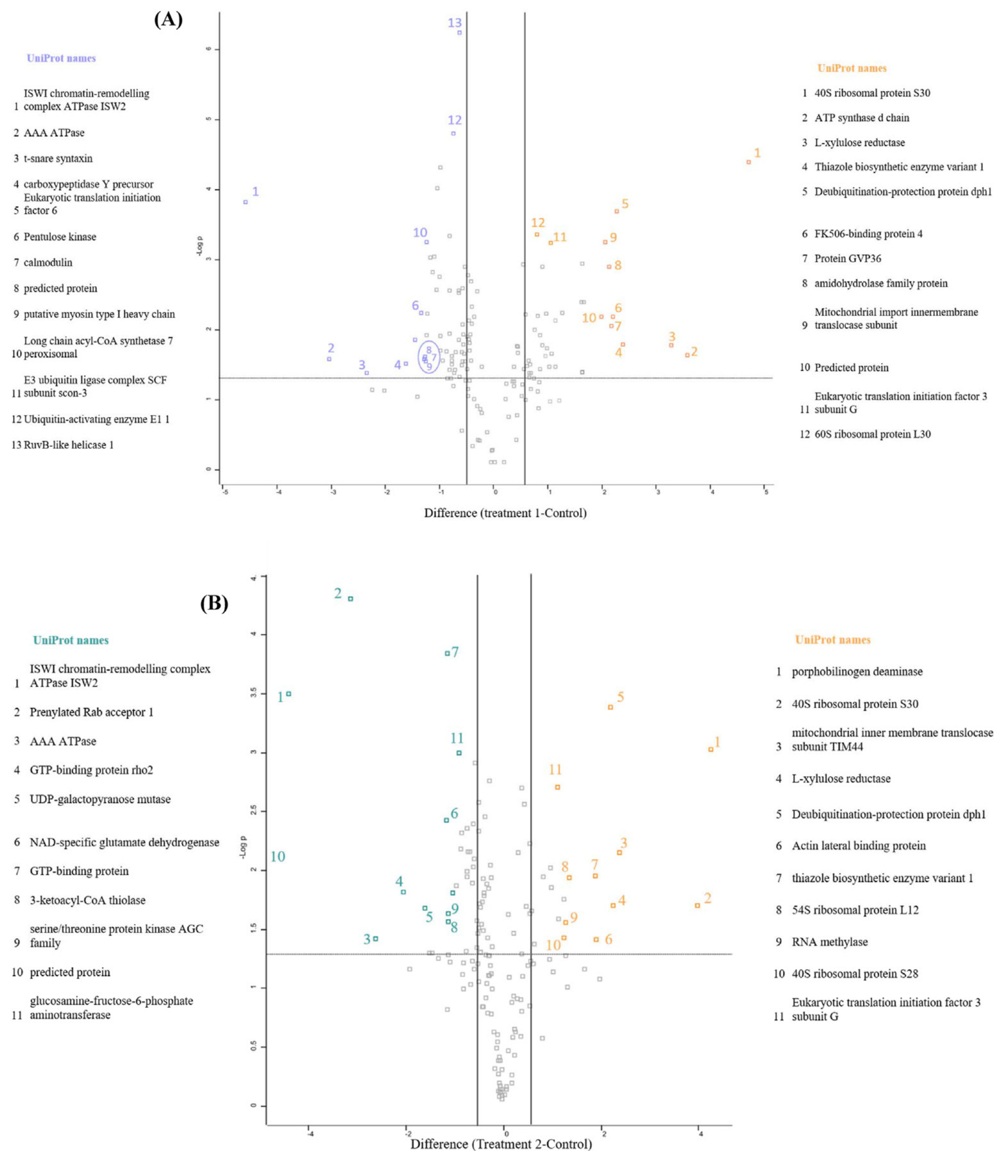


**Fig. 5.** Volcano plots display the distribution of statistically significant and differentially abundant (SSDA) proteins which have a -Log (p-value) >1.3 and difference ± 0.58.

One of the aims of this research was to identify the specific element within the *B. velezensis* culture filtrate which inhibited *C. mycophilum* growth. The results demonstrated that the inhibitory component was >3 kda. Qualitative proteomic analysis on the >3 kda, polar sample of 96 hr B. *velezensis* CF revealed a number of proteins which were present within this sample including peptidase S8 (subtilisin). Subtilisin was first isolated in *Bacillus subtilis* species, but has since been isolated from a number of *Bacillus* species (Ottesen and Svendsen 1970). This protein has serine-type endopeptidase activity and is known to be highly efficient at protein degradation. Its degradation abilities are so efficient that it is used as a key active ingredient in laundry detergents for the removal of proteinaceous stains (Vojcic et al., 2015). Genome mining has shown that *B. velezensis* species contain biosynthetic gene clusters within their genome which are responsible for the production of a number specialised metabolites, including subtilisin which contribute to their antimicrobial properties (Fazle Rabbee and Baek 2020; Mullins et al., 2020). Therefore it is possible that subtilisin released in the *B. velezensis* culture filtrate is responsible for the inhibition of *C. mycophilum* growth. The addition of purified subtilisin to *C. mycophilum* cultures slightly reduced the biomass growth of the fungus. This suggests that although subtilisin may be inhibitory, to see the level of inhibition achieved by the 96 hr B. *velezensis* CF, it may need to work in combination with other metabolites within in the CF. Probable cytosol aminopeptidase and peptide deformylase which are both involved in catalytic activity were also found within the sample (Hernick and Fierke, 2010). Future work, using HPLC fractionation will search to identify the specific proteins, either individually or in combination, which allow *B. velezensis* CF to inhibit the growth of *C. mycophilum*.

Kosanovic et al. (2021), examined the response of *T. aggressivum* to the same *B. velezensis* strain investigated in this study and found that, against *T. aggressivum*, zones of inhibition could only be produced when *B. velezensis* cells were applied to plates and no inhibition was recorded around culture filtrate samples. To the best of our knowledge, this is the first time that a *B. velezensis* culture filtrate has caused inhibition against a fungal plate culture. This opens up the possibility of applying the culture filtrate directly onto *A. bisporus* crops as a treatment method, rather than applying *B. velezensis* cells which is done with Serenade.

Proteomic analysis further supports the finding that *B. velezensis* culture filtrate causes significant growth inhibition of *C. mycophilum*. Principal component analysis, hierarchical clustering and a heatmap generated from the proteomic data indicate a clear difference between treatment and control samples. Volcano plots highlighted individual proteins which were SSDA. The majority of proteins which were downregulated in both treatment samples are structural components of the ribosome and were involved in translation. Essential growth processes such as translation appear to be significantly reduced in the presence of *B. velezensis* culture filtrate. ATP activity, ubiquitination, proteolysis, DNA repair and oxidation–reduction activity were all upregulated in both treatments, compared to the control samples. This would suggest that the *B. velezensis* culture filtrate is having a detrimental effect on *C. mycophilum,* as the activities which have increased in response to its presence are associated with cellular stress and growth inhibition.

Overall, activities associated with normal cellular growth (e.g. ribosomal/translation proteins) appeared to be downregulated in the treated samples. Similar proteins, involved in translation were also reduced when this *B. velezensis* culture filtrate was applied to *T. aggressivum* (Kosanovic et al., 2021). Proteins associated with cell stress response e.g. oxidative reduction, ATP activity, DNA damage were all increased in the samples exposed to *B. velezensis* culture filtrate compared to the control. Oxidoreductase and DNA repair proteins were also found to be increased in abundance in *T. aggressivum* treated with *B. velezensis* (Kosanovic et al., 2021). Blast2go GO analysis further confirmed that these particular activities were altered in treatment/control.

The results presented here indicate that *B. velezensis* culture filtrate is capable of inhibiting the growth of *C. mycophilum in-vitro* and of inducing a stress response. This confirms its potential as a biocontrol agent which could be used for the treatment of cobweb disease on mushroom growing farms and may offer an alternative to chemical fungicides for disease treatment. The number of approved fungicides which have been phased out by government bodies and environmental agencies has increased over the past two decades. Furthermore, the emergence of strains which are resistant to chemical fungicides have greatly limited their use. Continued use of chemical fungicides may exacerbate the problems faced by mushroom growers (Grogan and Gaze, 2000; Grogan, 2006; Potocnik et al., 2015). It is important that effective biocontrol methods are developed to replace fungicide use in the future. Serenade (*B. velezensis* QST 713) was approved 21 years ago in the USA and has been established as a biocontrol option for diseases of several cultivated crops, however there are few biocontrol options on the market. The development of resistant strains against Serenade is a worrying possibility (Marrone, 2002). This work will go towards the development of this *B. velezensis* strain as another biocontrol option for growers. *Bacillus* species are found naturally in the mushroom casing and are generally regarded as safe (GRAS). Kosanovic et al. (2021) have also shown in their work, that the *B. velezensis* strain used during this research does not negatively affect the growth of *A. bisporus.* Further experimental work is needed to establish if this strain has potential as a biocontrol agent and we will examine the response of *C. mycophilum/A bisporus* to *B. velezensis* culture filtrate in a crop trial in the future.

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## Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.funbio.2021.09.003.

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