Eur J Plant Pathol (2021) 160:397–409
https://doi.org/10.1007/s10658-021-02252-5

Differential proteomic response of *Agaricus bisporus* and *Trichoderma aggressivum* f. *europaeum* to *Bacillus velezensis* supernatant

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Accepted: 3 March 2021 / Published online: 10 March 2021

Abstract *Trichoderma aggressivum*, a mycopathogen causing green mould disease, is a major problem in *Agaricus bisporus* cultivation due to crop loss, and resistance to chemical fungicides. There is an urgent need for novel biological ways to control mycopathogens without affecting the growth of *A. bisporus*. Bacteria from the mushroom-casing environment were identified and tested for antagonistic effect on *T. aggressivum*. *Bacillus velezensis* produced a large zone of inhibition and its supernatant inhibited the growth of *T. aggressivum* [−37%], and slightly stimulated *A. bisporus* growth [+2%]. Label free quantitative-proteomic (LFQ) analysis of changes in the abundance of *T. aggressivum* proteins following exposure to *B. velezensis* supernatant indicated increased abundance of proteins associated with catabolic processing of amino acids (40-fold), amino oxidase proteins (14-fold), oxidoreductase proteins (13-fold, 4-fold) and hydrolases (3-fold). Proteins that decreased in relative abundance were antioxidants (29-fold), NTF2 domain containing protein (17-fold), 60S ribosomal protein L-13 (14-fold), glucoamylase proteins (13-fold), proteasome subunit proteins (11-fold) and other ribosomal proteins (9-fold). LFQ analysis revealed that exposing *A. bisporus* to *B. velezensis* supernatant led to a decrease in: prohibitin (13-fold, 6-fold), proteasomal proteins (11-fold), cytosolic adaptor domain containing protein (5-fold), aldehyde dehydrogenase (4-fold), ribosomal proteins (4-fold), DLH domain-containing protein (4-fold) and PKS_ER domain containing protein (3-fold). The results indicate that *A. bisporus* was not under stress upon contact with *B. velezensis*. Whereas a detrimental effect of *B. velezensis* on *T. aggressivum* is shown by inhibition of growth and damage-preventing proteins and increased abundance of proteins associated with stress.

Keywords *Agaricus bisporus* · *Trichoderma aggressivum* · *Bacillus velezensis* · Proteomics

Abbreviations

FDR	False Discovery Rates
GO	gene ontology
SSDA	statistically significant differentially abundant
DEP	differentially expressed proteins
LFQ	Label free quantitative-proteomic
ME	malt extract
CYM	complete yeast media
SN	supernatant
MPN	Most probable number
SE	Standard error

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Introduction

The world mushroom industry is dominated by five species: shiitake (*Lentinula* sp.) (20%), oyster mushrooms (*Pleurotus* spp.) (19%), wood ear mushroom (*Auricularia* spp.) (17%), common or white button mushroom (*Agaricus bisporus* (Lange) Imbach) (15%) and Enoki mushrooms (*Flammulina velutipes* (Curtis) Singer) (11%) with China accounting for 87% of total production (Royse et al. 2017). Outside of Asia, *A. bisporus* is the dominant species grown in North America and Europe (Royse et al. 2017) and the main horticultural crop in the Republic of Ireland (Alexander 2015). Cultivation of *A. bisporus* relies upon the use of a hybrid strain that was developed in 1980s, the Horst-U1 strain varieties. Thus, there is a lack of genetic diversity worldwide, making the global crop susceptible to attack from a variety of pathogens (Savoie et al. 2013).

Two sub-species of *T. aggressivum* (Samuels & W Gams): *T. aggressivum* f. *europaeum* (biotype Th4) and *T. aggressivum* f. *aggressivum* (biotype Th2), are responsible for causing green mould disease of *A. bisporus*, which is visible as a green sporulating mould on the mushroom substrate surface (Kosanović et al. 2013). *T. aggressivum* can destroy a mushroom crop if it gets into freshly spawned Phase II compost. The pathogen is extremely well-suited to the mushroom growing conditions at this stage of production. It is fast growing when presented with carbohydrate-rich substrates and it is well suited to the growing temperatures of *A. bisporus* (Grogan 2008).

Mycopathogens such as green mould on mushroom crops are challenging to treat because both pathogen and its host are fungal (Grogan 2006; Potočnik et al. 2015). A limited number of chemical fungicides are officially approved for use against mushroom diseases, depending on local pesticide regulations, and currently include: prochloraz, metrafenone, thiabendazole and chlorothalonil (Grogan 2008; Kosanović et al. 2015; Potočnik et al. 2015). The continuous usage of fungicides as a disease control strategy on mushrooms farms can lead to the development of pathogen resistance and host sensitivity (Fletcher and Yarham 1976; Grogan and Gaze 2000; Romaine et al. 2005; Gea et al. 2005; Grogan 2006; Potočnik et al. 2015). Pesticide use worldwide is under scrutiny due to concerns about their impact on biodiversity, the environment and human health. In the EU, pesticide use is currently regulated by the Sustainable Use of Pesticides Directive (SUD) 2009/128/EC (Anon 2009), which advocates an integrated pest management (IPM) approach to pest and disease control. This involves many practices including pest prevention and monitoring, using sustainable biological or non-chemical methods where possible and using anti-resistance strategies to maintain effectiveness of products. There is an urgent requirement for more biological control products for the mushroom sector. Serenade® is a widely used bio-fungicide for many years containing non-pathogenic *Bacillus velezensis* strain QST 713 (Kosanović et al. 2013; Pandin et al. 2018) while Serifel® is a more recent product based on *Bacillus amyloliquefaciens* strain MBI600, both are approved for use against the mushroom pathogen *Trichoderma aggressivum* but there is little information on how they interact with the host and pathogen to achieve control (Kosanović et al. 2013).

Proteomics, the study of proteins and protein interactions in a cell or organism, is an advanced technology that allows a greater understanding of disease states in combination with bioinformatics. This technology can be used to give an insight into what is occurring upon contact between the host and the pathogen on the proteome level (Cho 2007). By identifying particular proteins that have been either over-expressed or under-expressed as a result of this contact, and determining affected biochemical pathways, it is possible to begin to build a picture of the exact mechanisms underlying the pathogenicity or defence mechanisms of a cell.

Since naturally occurring bacteria and fungi coexist and compete within the mushroom growing substrate, native *Bacillus* spp. are considered as potential alternatives to chemical control of green mould disease (Védie and Rousseau 2008; Kosanović et al. 2013; Potočnik et al. 2019). This paper details the testing of a supernatant derived from an environmental isolate of *Bacillus velezensis* as a potential biocontrol agent against green mould disease in *A. bisporus* cultivation. Bacteria supernatants, also known as culture filtrates, contain the microbial secretome with a range of toxins, enzymes and other products secreted by microbes that may play a role in pathogenesis (Mc Namara et al. 2017; Kosanović et al. 2019).

The aim of the work presented here was to characterise the proteomic interactions between (i) *A. bisporus* and *B. velezensis*, and (ii) *T. aggressivum* and *B. velezensis*.

Material and methods

Fungal cultures

T. aggressivum f. *europaeum* 100,526 (acquired from the Westerdijk Institute, Royal Netherlands Academy of Arts and Science, Ultrecht, Netherlands) preserved in liquid nitrogen culture collection of Maynooth University and *A. bisporus* commercial strain A15 (Sylvan Inc., France) were used in this study. *Trichoderma* stocks were maintained on malt extract (ME) agar (Oxoid). *A. bisporus* was maintained as spawn (sterilized rye grains colonised with *A. bisporus*) and on CYM agar plates.

Bacterial cultures

Algoriphagus sp., *Sphingopyxis* sp., *Nocardioides* sp., *B. velezensis* (isolated from mushroom growing substrate - Teagasc, Ireland), *B. subtilis* MU Prep lab and *B. velezensis* QST 713 (Serenade® - Bayer) were used in this study. Bacterial stocks were maintained on nutrient agar (NA) (Oxoid).

Isolation and identification of bacteria from mushroom casing in a production environment

Bacteria were isolated from mushroom casing, a mixture of peat and lime (Harte Peat Ltd., Ireland), taken from a crop in cultivation at the Teagasc Mushroom Growing Unit (Ashtown, Dublin, Ireland). Compost bacteria are known to migrate from compost to casing (Grewal and Rainey 1991). Also, growers sometimes mix some compost into the casing (CAC technique-Compost Added at Casing) (MacCanna and Flanagan 1972) and thus increase the process of casing colonization via microorganisms (at Teagasc Mushroom growing facility the same technique has been performed). Isolates were maintained on nutrient agar (NA) (Oxoid). 16S rRNA gene sequencing was used to identify the selected colonies. PCR analysis was carried out with V3 and V4 region of bacterial 16S rRNA, using forward: 5'TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGCCTACGGGNGGCWGCAG3' and reverse: 5'GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGGACTACHVGGGTATCTAATCC3' primers (Klindworth et al. 2013) for amplification of the gene. Each 50 μL reaction mixture consisted of 10 μl Reaction buffer, 1 μl of 10 μM forward and reverse primers, 0.5 μl of Taq polymerase, 3 μl of sample and 34.5 μl of dH_2O. A sample free (dH_2O substitute) reaction mixture was used as a negative control. Initial denaturation of samples was carried out at 95 °C for 3 min, followed by 35 cycles of denaturation at 95 °C, primer annealing at 55 °C and extension at 72 °C all for 30 s with the final extension at 72 °C for 5 min. Five μl of PCR products were separated by electrophoresis in 1% agarose gel, stained with SYBR safe and visualized with a G-BOX (Syngene, UK). The clean-up of PCR products was carried out on a magnetic plate using KAPA Pure Beads (Roche), washed two times in 70% Ethanol and resuspended in Elution buffer. The concentration and quality of isolated DNA was evaluated using a DeNovix DS-11 spectrophotometer (A260/A280 ratio). Sequencing was performed by Eurofins Genomics Sequencing GMBH (Germany). The obtained sequences were analyzed using BLAST (https://blast.ncbi.nlm.nih.gov/Blast.cgi) and prepared for deposition to NCBI (National Centre for Biotechnology Information) GenBank database by BioEdit program. GenBank accession numbers for deposited nucleotide sequences were assigned (Table S1).

Additional sequencing was performed to verify the identity of *B. velezensis,* 16S rRNA whole gene analysis and gyrB gene analysis by Charles River laboratory (France) (Fig. S1 & S2).

Antagonistic assay

Ten microliter drops of a bacterial overnight broth culture were transferred onto NA and YMEA (yeast malt extract agar), 2 g L^{-1} yeast extract, 20 g L^{-1} malt extract, 15 g L^{-1} agar, (Pandin et al. 2018) plates inoculated with 10^4 *T. aggressivum* conidia and incubated for 48 h at 25 °C. Following this, the plates were examined for zones of inhibition around the 10 μl bacterial culture drops. Nutrient broth (NB) was used as a negative control.

Assessment of effect of bacterial supernatants on wet weight of *T. aggressivum*

Bacterial cultures were grown in nutrient broth (NB) (Oxoid) at 30 °C until the stationary phase was reached, then centrifuged and filter-sterilized through 0.2 μm syringe filters to produce supernatants. Cultures (50 ml) of *T. aggressivum* 10^4 conidia/ml were grown

for 24 h at 30 °C and 200 rpm in ME broth and then supplemented with bacterial (*Algoriphagus* sp., *Sphingopyxis* sp., *Nocardioides* sp., *Bacillus* spp.) stationary phase supernatant to give a final concentration of 25% *v*/v and incubated for an additional 24 h under the same conditions. Cultures were harvested by centrifugation at 4143×g for 15 min. The supernatant was discarded, and wet mass was measured.

Effect of *B. velezensis* supernatant on *A. bisporus* growth

Cultures (50 ml) of *A. bisporus* (one spawn grain of A15 in CYM broth) were grown for 8 days in CYM broth at 25 °C and 200 rpm and supplemented with 25% v/v bacterial supernatant for an additional 2-days at 25 °C. *A. bisporus* mycelium was harvested by centrifugation at 4143×g for 15 min. The supernatant was discarded, and wet mass was measured.

LFQ proteomics of *T. aggressivum* and *A. bisporus* treated with *B. velezensis* supernatant

Proteins were extracted according to Maher et al. (2018) from *A. bisporus* mycelium which was grown for 8-days and then supplemented with *B. velezensis* supernatant ($n = 3$) for an additional 2-days. Proteins were also extracted from *T. aggressivum* mycelium which was grown for 24 h and then supplemented with *B. velezensis* supernatant (n = 3) for an additional 24 h. The Bradford method was used to quantify proteins for acetone precipitation overnight. Samples were centrifuged at 14500×g for 10 min, and the pellet was resuspended in 25 µl of 6 M urea, 2 M thiourea and 0.1 M Tris-HCl buffer (pH 8.0). Proteins were reduced with dithiothreitol (0.5 M DTT), alkylated with iodoacetamide (0.55 M IAA) and digested with sequence grade trypsin (Promega, Ireland) at a trypsin: protein ratio of 1:40, overnight at 37 °C. Tryptic peptides were purified for mass spectrometry using C18 spin columns (Medical Supply Company, Ireland) and 0.75 µg of peptide mix was eluted onto 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%–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. Identification of proteins from this data was performed using the Andromeda search engine in Max-Quant (version 1.2.2.5) to correlate against an *A. bisporus* and *T. aggressivum* database downloaded from www.uniprot.org.

The MS proteomic data and MaxQuant search output files have been added to the ProteomeXchange Consortium (Perez-Riverol et al. 2019) via the PRIDE partner repository with the dataset identifier PXD017970. The Perseus software package (v. 1.5.5.3) was used for results processing, statistical analyses and graphics generation. LFQ intensities were log2-transformed and ANOVA of significance and t-tests between the treated groups was performed using a p value of 0.05 and significance was determined using FDR correction (Benjamini and Hochberg 1995). Proteins which had non-existent values (suggestive of absence or very low abundance in a sample) were also used in statistical analysis. Proteins found to be absent (below the level of detection) in one or more treatments and present (above the level of detection) in three or fewer treatments were also used in statistical analysis of the total differentially expressed group following imputation of the zero values with values that simulate low abundant proteins.

These proteins were subjected to Gene Ontology (GO) analysis by Blast2GO software tool (https://www.blast2go.com/). Statistically enriched GO descriptors were examined to identify clusters of proteins enriched within statistically significant differentially abundant (SSDA) protein lists ($p < 0.05$).

The Search Tool for the Retrieval of INteracting Genes/Proteins (STRING) v10.5 (http://string-db.org/) was used to map known and predicted protein:protein interactions. UniProt gene lists (extracted from Perseus) were inputted and analyzed in STRING using the medium confidence (0.5) setting to produce interactive protein networks for proteins increased and decreased in abundance.

Statistical analysis

Proteomic and yield experiments were carried out in three replicates and results are expressed as the mean ± SE. ANOVA and t-tests with significant differences were considered at $p < 0.05$ (Prism 5.0, GraphPad Software, Inc.).

Results

Bacterial identification

A range of bacteria was isolated from the mushroom casing and cultivated on NA (Oxoid). Four individuals were selected for further study: *Algoriphagus* sp., *Sphingopyxis* sp., *Nocardioides* sp. and *B. velezensis*. Bacterial amplicons were deposited in GenBank database and analyzed by BLAST program (Table S1). *B. velezensis* QST 713 (Serenade® - Bayer) and a *B. subtilis* isolate from Maynooth University Prep Laboratory were also included in this study. *B. velezensis* (Fig. S1 & S2) deposited in GenBank under MT156336, is the main focus of this study.

Antagonistic test

B. velezensis produced a zone of inhibition (radius = 8.2 mm) on NA agar plates inoculated with 10^4 *T. aggressivum* conidia after 48 h (Fig. S3a). Two of the mushroom casing bacteria identified in this study had no inhibitory effect, while *Sphingopyxis* sp., *B. velezensis* QST 713 and *B. subtilis* (MU Prep Lab) produced zones of inhibition of 36.18 ± 2.2, 67.86 ± 5.1 and 32.8 ± 2.9 mm^2, respectively when incubated on NA plates (Fig. S3a). Further tests performed on YMEA and NA plates at 25 °C showed that *B. velezensis* and *B. velezensis* QST 713 produced an area of inhibition 196.6 ± 19.5 mm^2 and 309.0 ± 46.0 mm^2 on YMEA, respectively (Fig. S3b). *B. subtilis* produced no area of inhibition on YMEA or NA media (Fig. S3b).

Effect of *B. velezensis* supernatant on *T. aggressivum* growth

The effect of bacterial supernatants on *T. aggressivum* growth was assessed after 48 h by measuring the wet mass of the *T. aggressivum* mycelium. The *B. velezensis* supernatant (25%v/v) produced the greatest inhibition of *T. aggressivum* wet mass (Fig. S4) and in a subsequent trial containing *B. velezensis* supernatant *T. aggressivum* growth was 2.9 ± 0.3 g compared to the control 4.6 ± 1.0 g ($P = 0.15$, df = 6, t = 1.7) (Fig. 1a). Since the *B.velezensis* isolate produced the greatest inhibition of mycelial growth it was selected for subsequent analysis.

Effect of *B. velezensis* supernatant on *A. bisporus* growth

The effect of *B. velezensis* supernatant on *A. bisporus* growth was assessed after an initial 8-day incubation of *A. bisporus* at 25 °C and an additional 2-days of co-incubation with 25% v/v *B. velezensis* filter-sterilized supernatant or NB (control). *B. velezensis* supernatant did not show any effect on the growth of *A. bisporus* mycelia. Average wet mass for the control was 5.1 g ± 0.7 and for the treatment was 5.2 g ± 1.2 (Fig. 1b). There was no statistical difference between control and treatment samples ($P = 0.95$, df = 6, t = 0.07). The material was used for further proteomic analyses.

Proteomic analysis of response of *T. aggressivum* after exposure to *B. velezensis* supernatant

In total 9942 peptides were identified, representing 863 proteins, following exposure of *T. aggressivum* to *B. velezensis*. According to Perseus analysis a total of 417 *T. aggressivum* proteins in the *B. velezensis* treatment were determined to be differentially abundant (analysis of variance [ANOVA], $P < 0.05$), 89 increased and 328 decreased in relative abundance with a fold change value of >2.

A principal-component analysis (PCA) performed on all filtered proteins distinguished the control and *B. velezensis*-treated samples, indicating a clear difference between the proteomes (Fig. S5). *T. aggressivum* control group (unchallenged) and *T. aggressivum* treated with *B. velezensis* supernatant showed clear differences based on hierarchical clustering of proteins increased in one group and decreased in another (Fig. S6). The clear difference in protein abundance similarities based on hierarchical clustering between the control group of *T. aggressivum* vs *T. aggressivum* treated with *B. velezensis* supernatant is further highlighted in Fig. S6. This image shows a heat map constructed using the data gathered from label-free proteomic analysis of *T. aggressivum* control vs. *T. aggressivum* treated with *B. velezensis* supernatant. Indicating that specific bunches of proteins that are increased in abundance in one group are decreased in another and vice versa, further pointing to the influence that the supernatant had on the overall proteome compared to control.

Exposure of *T. aggressivum* to *B. velezensis* supernatant led to an increase in proteins associated with catabolic processing of amino acids (40-fold), amino

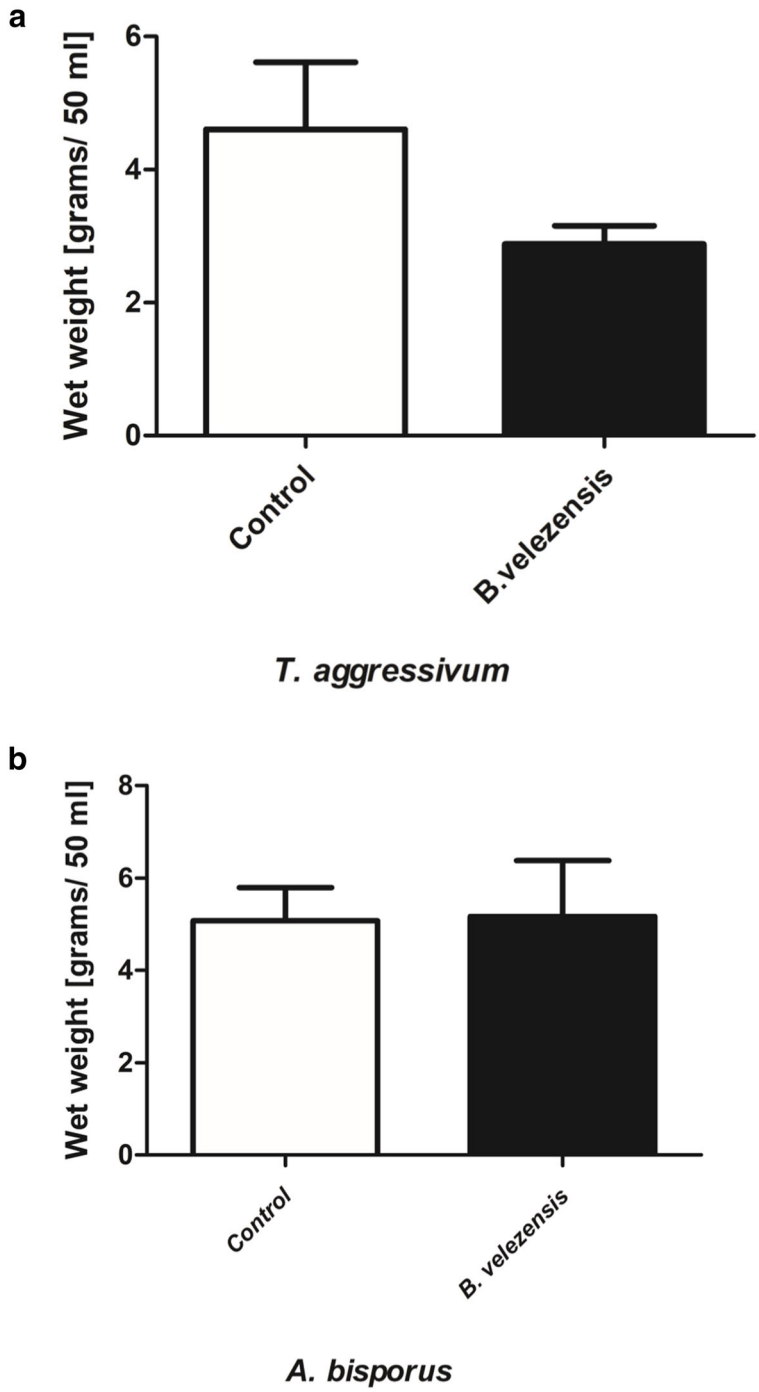

Fig. 1 **a.** *T. agressivum* growth [g] in CYM after 48 h incubation, containing 25% *v*/v of either: NB (Control) or *B. velezensis* supernatant. ($p = 0.15$, t = 1.66, df = 6). **b.** *A. bisporus* growth [g] in CYM after 8-days incubation at 25 °C, containing 25% v/v of either: NB (Control) or *B. velezensis* supernatant for the last 2-days of incubation. $P = 0.95$, t = 0.066, df = 6

oxidase proteins (14-fold), oxidoreductase proteins (13-fold, 4-fold) and hydrolases (3-fold). These results suggest that exposure to *B. velezensis* supernatant has a damaging effect on *T. aggressivum* as proteins associated with the breakdown of essential amino acids increased as did proteins associated with stress (Fig. 2).

In addition to this, exposure of *T. aggressivum* to *B. velezensis* supernatant led to a decrease in the abundance of proteins such as anti-oxidants (29-fold), NTF2 domain containing protein (17-fold), 60S ribosomal protein L-13 (14-fold), glucoamylase proteins (13-fold), proteasome subunit proteins (11-fold) and other

ribosomal proteins (9-fold). This further emphasizes the detrimental effect of *B. velezensis* on *T. aggressivum* as essential growth proteins and proteins associated with preventing damage in cells decline (Fig. 2).

According to STRING analysis (string-db.org) there was enrichment of protein groups in *T. aggressivum* sample exposed to *B. velezensis* supernatant (Fig. 3). Protein groups that were found to have more interactions among themselves were ribosomal, mitochondrial, catabolic, dehydrogenase and proteolytic proteins. Ribosomal proteins are involved in growth and development processes that seem to be altered in treated samples, and the mitochondrial cluster shows alteration in oxidase production meaning that the hyphae may have been under stress. Dehydrogenase proteins, and especially detoxification proteins, may be stimulated as a defense mechanism. Proteolysis breakdown of proteins into polypeptides or amino acids is also noted.

Proteins were subjected to Gene Ontology (GO) analysis by Blast2GO software tool (Fig. S7 a, b, c, d). Several GO terms belonging to biological process (oxidation-reduction and organic substance metabolic process), molecular function (ion binding, oxidoreductase activity), cellular component (intracellular organelle, membrane and cytoplasm) were significantly enriched within the *B. velezensis* group dataset. Hydrolases, oxidoreductases and transferases were the most enriched enzymes with hydrolases being the most abundant in *B. velezensis* challenged group. Indicating oxidative stress response.

Proteomic analysis of response of *A. bisporus* after exposure to *B. velezensis*

Following exposure to *A. bisporus* to *B. velezensis* supernatant a total 619 peptides were identified, representing 81 proteins. According to Perseus analysis a total of 12 *A. bisporus* proteins in the *B. velezensis* treatment were determined to be differentially abundant (analysis of variance [ANOVA], $P < 0.05$), 1 increased and 11 decreased in relative abundance with a fold change value of >2.

A principal-component analysis (PCA) performed on all filtered proteins distinguished the control and *B. velezensis* treated samples (Fig. S8). Even though difference between control and treatment group proteomes exists these groups are close, suggesting less alteration in proteins between control and treatment groups.

Exposure of *Agaricus bisporus* to *B. velezensis* supernatant led to a decrease in proteins such as Prohibitin which plays a role in cell survival (13-fold, 6-fold), proteasomal proteins (11-fold), signaling proteins such as cytosolic adaptor domain containing protein (5-fold), aldehyde dehydrogenase which plays a role in cell detoxification (4-fold), ribosomal proteins (4-fold), DLH domain-containing protein (4-fold) and proteins involved in secondary metabolism such as PKS_ER domain containing protein (3-fold) (Fig. 4). The down-regulation of such proteins suggests that *A. bisporus* was not put under any stress upon contact with *B. velezensis* supernatant.

Only one protein was over-expressed in a statistically significant way (2-fold) but was found to be uncharacterized (Fig. 4). Combined, these results suggest that *B. velezensis* has no adverse effects on *A. bisporus,* a potentially useful result if *B. velezensis* was to be considered as a biocontrol against fungal pathogens of button mushrooms.

Discussion

Effective biocontrol agents are urgently required globally as many chemical fungicides are being withdrawn or phased out. Reasons include pathogens developing resistance, chemicals being deactivated in substrates, toxicity to crops, and toxicity to non-target organisms (Grogan and Gaze 2000; Romaine et al. 2005; Gea et al. 2005; Grogan 2006; Potočnik et al. 2015; Stanlet and Preetha 2016). Hence, there is an intense focus placed on the discovery of novel, superior biocontrol bacteria (Potočnik et al. 2019).

Serenade® is Bayer's biocontrol agent containing non-pathogenic *Bacillus velezensis* (originally identified as *B. subtilis*) strain QST 713 for the treatment of fungal diseases in agricultural crops. Only a limited number of similar products are available on the commercial market. It contains compounds with multiple mechanisms of action, which makes resistance less likely. *Bacillus velezensis* strain QST 713 releases compounds such as lipopeptides which penetrate cell membranes of pathogenic cells, causing the cells to become leaky, allowing the entry of other anti-fungal compounds and resulting in cell death (Marrone 2002).

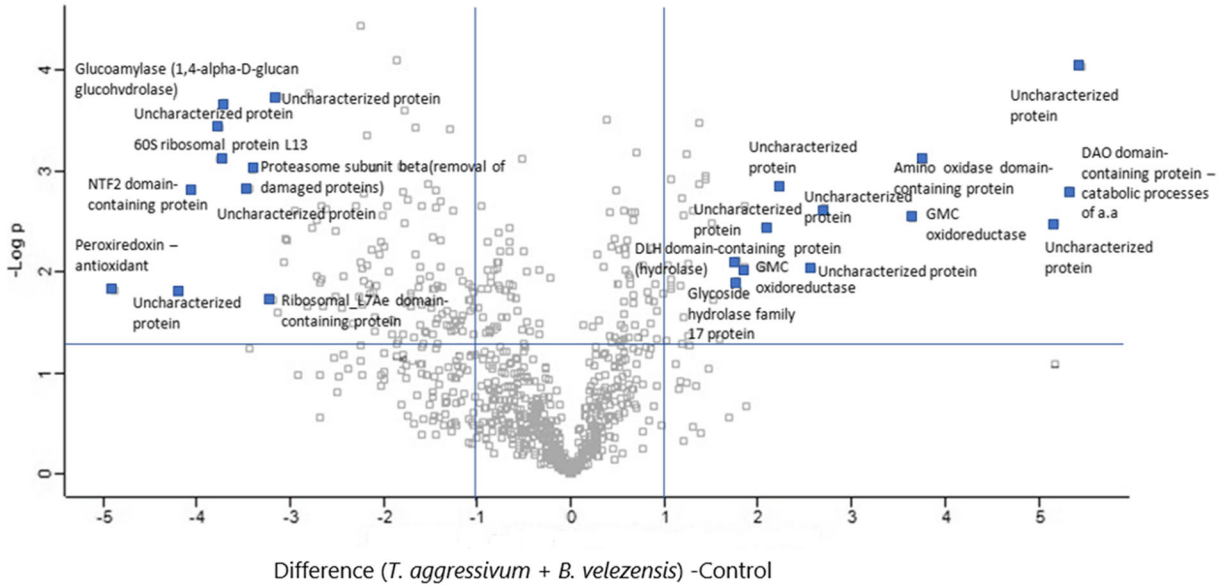

Fig. 2 Proteomic responses of *T. aggressivum* cultivated for 24 h and co-incubated with 25% v/v supernatant of. *B. velezensis* for additional 24 h. Volcano plot represents protein intensity difference (− log2 mean intensity difference) and significance in differences (− log *P* value) based on a two-sided t-test. Proteins above the line are considered statistically significant (*p* value <0.05) and those to the right and left of the vertical lines indicate relative fold changes >2. Annotations are given for the most differentially abundant proteins identified. These plots are based upon post imputed data

Improvement of white mushroom yield following use of a *Bacillus* sp. has been reported by Tautorus and Townsley (1983). *Bacillus* sp. added to *A. bisporus* compost significantly improved the mycelial growth and was effective with olive green mould (*Chaetomium olivaceum*) control. Védie and Rousseau (2008) described efficacy of Serenade® against *Trichoderma* sp. Recent studies also showed the efficacy of *B. velezensis* in vivo trials (Pandin et al. 2018). Elsewhere, Nagy et al. (2012) observed that *B. amyloliquefaciens* (species used in Serifel®) was effective in vivo against *T. pleurotum*, the causal agent of green mould on oyster mushroom, and that it improved the mushroom yield (+10%). Kosanović et al. (2013) found that *B. velezensis* QST 713 (strain used in Serenade®) was highly toxic (in vitro) to all tested *Trichoderma* isolates from mushroom growing sites (ED50 values were below 1.3 mgL^{-1}): *T. aggressivum* f. *europaeum*, *T. harzianum*, *T. atroviride*, *T. koningii* and *T. virens*. In a subsequent in vivo trial, a mixture of fungicide and biocontrol agent (80:20, prochloraz: Serenade®) was applied in the mushroom growing room and an antagonistic interaction was found between prochloraz and Serenade® regarding mushroom productivity. Disease symptoms were better prevented with individual treatments compared to their mixtures. Therefore, Serenade® showed better disease control when it was applied alone than its mixture with the fungicide. However, Serenade® decreased mushroom yield in this trial compared to the controls (uninoculated and inoculated with *T. harzianum*) and the prochloraz fungicide treatment (Kosanović et al. 2013).

Bacillus species are generally considered as effective biocontrol agents due to inhibitory metabolites such as cell-wall degrading enzymes (Cawoy et al. 2011; Khan et al. 2018), their growth-promoting plant hormones (Akinrinlola et al. 2018; Radhakrishnan et al. 2017; Sansinenea 2019) and ability to induce the equivalent of plant adaptive immunity (Fira et al. 2018). Many *Bacillus* species display antibiosis - an antagonistic effect towards pathogens (Kim and Chung 2004; Leelasuphakul et al. 2006; Živković et al. 2010; Cawoy et al. 2011; Bacon et al. 2015). *Bacillus* spp. produce spores that are resistant to various physical and chemical treatments such as heat, desiccation, UV irradiation and organic solvents (Leelasuphakul et al. 2008).

There are major concerns around the treatment of mycopathogens in the mushroom industry as host and pathogen are fungal so any biocontrol agent must show a differential effect in order not to compromise mushroom yield but still control the pathogen. Some *Trichoderma* spp. are used as biocontrol agents in plant

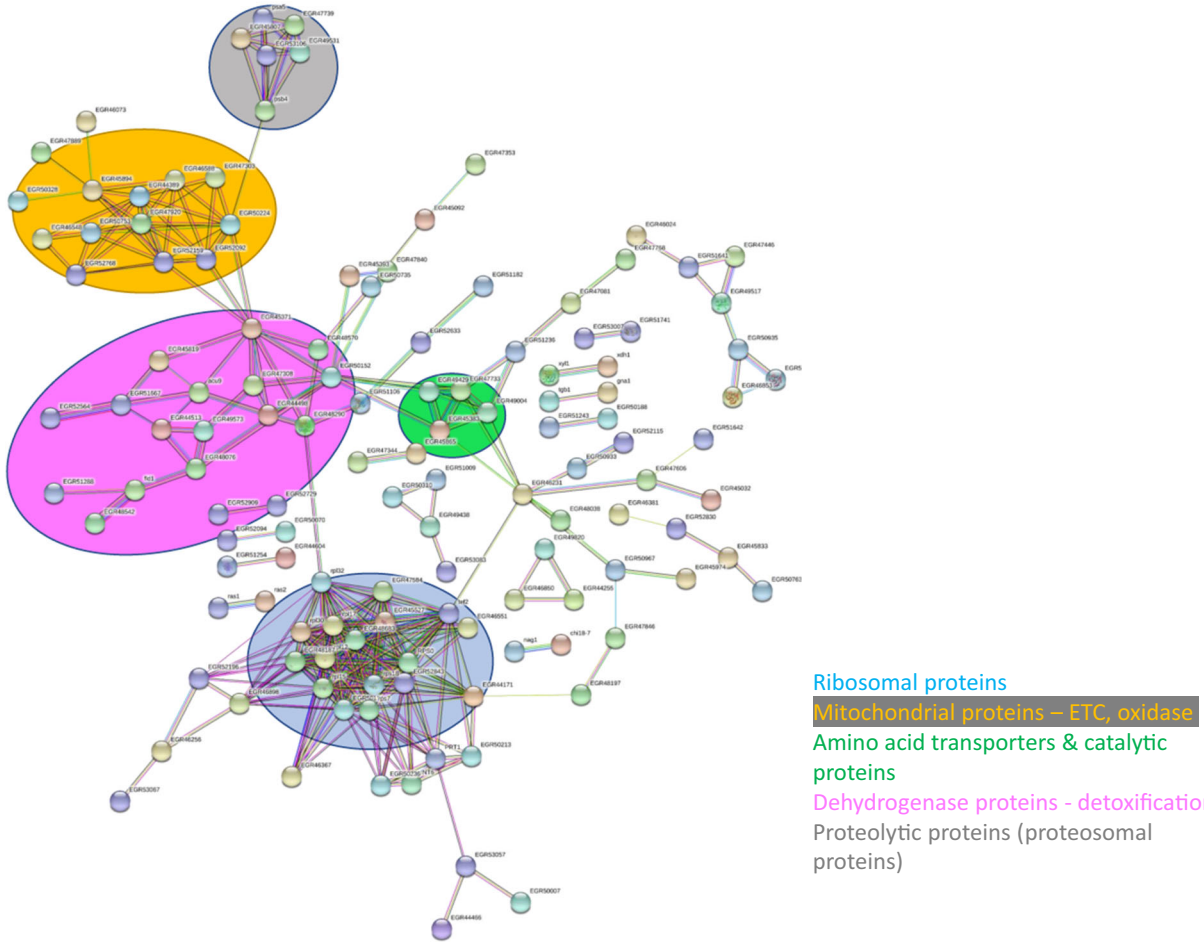

Fig. 3 STRING analysis showing enrichment of protein groups seen in *T. aggressivum* sample treated with *B. velezensis* that have more interactions among themselves

protection (Weindling 1932; Harman et al. 2010; Lorito et al. 2010), but while they may be beneficially used for the control of fungal pathogens of plant-based crops, they are not desirable in mushroom crop production since, not only *A. bisporus* but Oyster mushroom (*Pleurotus ostreatus*) and Shiitake (*Lentinula edodes*) are also prone to *Trichoderma* infections (Qiu et al. 2017; Wang et al. 2016).

Screening of a variety of bacteria from mushroom compost (*Algoriphagus* sp., *Sphingopyxis* sp., *Nocardioides* sp., *Bacillus* sp) and comparing with commercial bacteria (*B. velezensis* QST 713, Serenade® - Bayer) led to identification of one *Bacillus* sp., *B. velezensis*, that inhibited *T. aggressivum* growth and did not affect *A. bisporus*.

Isolated *B. velezensis* produced the highest zone of inhibition on NA plates (radius = 8.2 mm, Fig. S3) and its supernatant inhibited the biomass production of *T. aggressivum* (Fig. 1a**, − 37%**; Fig. S4). A principal-component analysis distinguished the control and *B. velezensis* -treated samples, indicating a clear difference between the proteomes (Fig. S5). The Heat map showed that proteins increased in abundance in control are decreased in *T. aggressivum* treated with *B. velezensis* and vice versa, pointing to the influence of *B. velezensis* supernatant on *T. aggressivum* proteome (Fig. S6). Further, label free proteomic analysis indicated that *T. aggressivum* showed an increased abundance in stress response proteins, oxidoreductase (14-fold, 13-fold and 4-fold) and hydrolase (3-fold), and a strong increase in abundance of proteins associated with catabolic processing of essential amino acids (40-fold) (Fig. 2).On the other hand, exposure of *T. aggressivum* to *B. velezensis* supernatant led to a

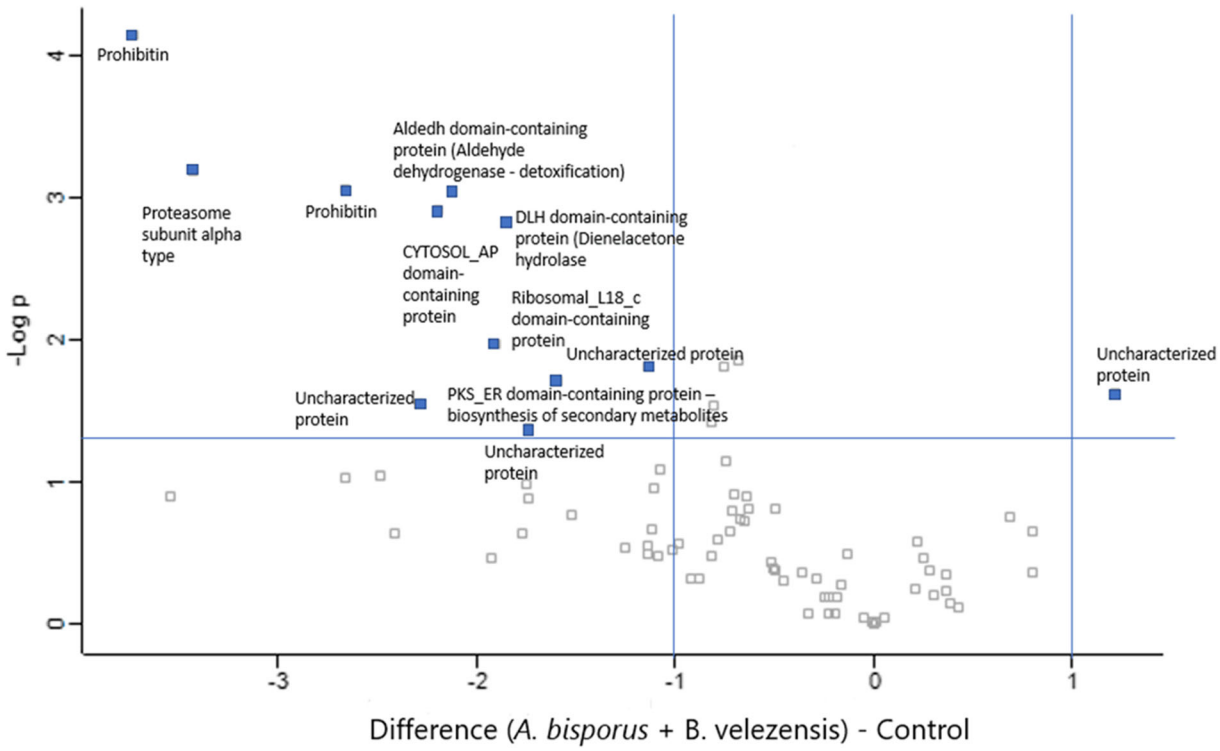

Fig. 4 Proteomic responses of *A. bisporus* cultivated for 8 days and co-incubated with 25% v/v supernatant of *B. velezensis* for additional 48 h. Volcano plot represents protein intensity difference (− log2 mean intensity difference) and significance in differences (− log P value) based on a two-sided t-test. Proteins above the line are considered statistically significant (p value <0.05) and those to the right and left of the vertical lines indicate relative fold changes >2. Annotations are given for the most differentially abundant proteins identified. These plots are based upon post imputed data

reduction in the abundance of proteins associated with growth such as 60S ribosomal protein L-13 (14-fold) and other ribosomal proteins (9-fold). Also, antioxidants (29-fold), NTF2 domain containing protein (17-fold), glucoamylase proteins (13-fold) and proteasome subunit proteins (11-fold) were decreased (Fig. 2). Gene Ontology analysis supported oxidative stress response in *B. velezensis* treated group (Fig. S7 a, b, c, d). While, STRING analysis showed ribosomal proteins, mitochondrial stress response, detoxification defense mechanism and catabolic processes of proteins, polypeptides and amino acids to be active (Fig. 3). All these results indicate that exposure of *T. aggressivum* to *B. velezensis* supernatant cause growth inhibition of mycopathogenic *T. aggressivum* and the induction of an oxidative stress response in the fungal cells.

In contrast, exposure of *A. bisporus* to *B. velezensis* supernatant showed no effect on *A. bisporus*. Biomass growth was slightly stimulated after co-incubation with *B. velezensis* supernatant (Fig. 1b, **+ 2%**). A principal-component analysis performed on all filtered proteins distinguished the control and *B. velezensis* -treated samples but also found this group proteomes are very close (Fig. S8). LFQ proteomic examination shows that *A. bisporus* was not under stress upon contact with *B. velezensis* supernatant (Fig. 4) – no decrease in growth and no stress induced proteins were evident. Exposure of *A. bisporus* to *B. velezensis* supernatant has led to a decrease in protein which plays a role in cell survival: prohibitin (13-fold, 6-fold), secondary metabolism: PKS_ER domain containing protein (3-fold) and cell detoxification: aldehyde dehydrogenase (4-fold) (Fig. 4). The down-regulation of such proteins, suggests that *A. bisporus* was not under stress upon contact with *B. velezensis* supernatant.

The results obtained by an in vitro experimental design may not reflect the *A. bisporus* crop environment and further in vivo trials with the culture supernatant are essential to potentially confirm the in vitro results. The use of the culture supernatant, instead of the whole

bacterium, might be beneficial since it does not depend on survival of the bacterium within the microbial community of the cropping system.

Conclusion

B. velezensis showed favourable characteristics to be considered as a future biocontrol agent as it affects the mycopathogen (*T. aggressivum*) but does not produce any damage to the white mushroom (*A. bisporus*). Further testing of *B. velezensis* supernatants is required, both in mushroom growing test unit and in laboratory, to identify, purify and concentrate the specific components of the secretomes that play a role in pest control. In vivo trials with the culture supernatant are essential to potentially confirm the in vitro results.

Supplementary Information The online version contains supplementary material available at https://doi.org/10.1007/s10658-021-02252-5.

Acknowledgments DK is a Postdoctoral Fellow supported by Irish Research Council, GOIPD/2018/115. Q-Exactive mass spectrometer was funded under the SFI Research Infrastructure Call 2012; Grant Number: 12/RI/2346. Dr. Thi Thuy Do, Maynooth University Antimicrobial Resistance and Microbiome Research Group, for helping with DNA extraction, sequencing, and identification.

Declarations This article does not contain any study with human participants or animals.

Conflict of interest The authors have no conflicts of interest to declare.

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