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Mushroom exo-polysaccharides as control agents of aflatoxin and ochratoxin A biosynthesis

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1. PhD Thesis

1.1 Introduction

Mycotoxins are hazardous secondary metabolites produced by wide range of saprophytic fungi able to contaminate foodstuffs such as cereals, dried fruits, nuts and spices (Bennett and Klich, 2003). Growth of fungi can occur either before harvest or after harvest, during storage, on/in the food itself often under warm, damp and humid conditions (Reverberi et al., 2010; Aiko and Mehta, 2015). According to Food and Agriculture Organization of the United Nations (FAO 1982), several hundred different toxic secondary metabolites identified, but the most important ones for which legislative limits exist because of human and animal health concerns include aflatoxins (AFs), ochratoxin A (OTA), patulin, fumonisins (FB), zearalenone (ZEA) and type A and B trichothecenes, including deoxynivalenol (DON) and T-2/HT-2 toxins. Due to their ubiquity in nature, these fungi are the most frequent pathogens of field crops, and also they are found in food and feed in a wide range of concentrations in different temperate and tropical climatic regions (Klarić et al, 2009). The molecules have no particular odor, taste or colour by which they presence in food or feed could be detected (Berndt, 1987). Moreover, some mycotoxins are heat resistant, and therefore cannot be removed by thermal processing of food commodities (Calado et al., 2014). Therefore, the mycotoxin contamination represent a great risk for human and animal health. Many mycotoxins were identified after they had caused a variety of health problem in humans and animals, with many target organs and systems affected (Magan and Olsen, 2004). Diseases caused by their toxicity in humans and animals are named mycotoxicosis. Acute mycotoxicoses occur during human and animal exposure to high concentrations of mycotoxins, with symptoms of severe illness appearing quickly after consumption of food products contaminated with mycotoxins (Peraica and Rašić, 2012). Acute mycotoxicoses are common in countries with tropical climates, where conditions for fungus development are more favorable (Peraica and Rašić, 2012). In temperate countries, large-scale acute toxicities are rare, although they have repeatedly affected the population of Europe several times in history. Chronically mycotoxicosis are the result of long-term exposure to a mycotoxin and have been linked to long-term effects on health, including the induction of cancers and immune deficiency (Peraica and Rašić, 2012). The association of disease onset with exposure to a mycotoxin is very difficult to prove, because humans and animals are simultaneously exposed to effect of different mycotoxins (Peraica and Rašić, 2012). The first mycotoxins were discovered in 1960s, with the outbreake of „Turkey-X disease“ in England. Turkey X disease

refers to the death of 100,000 Turkey poults as a result of acute mycotoxicosis due to consuming contaminated feed based peanut meal from Brazil (Blount, 1961). The fungus *Aspergillus flavus* was isolated, and its secondary metabolites were then named aflatoxins (Blount, 1961). There have been several outbreakes of mycotoxicosis during history in human population. In 1974, an outbreak of hepatitis in India caused the death of 100 people due to consumption of contaminated maize (Krishnamachari et al., 1975). The Kenyan outbreak in 2004 was one of the largest outbreakes, resulting in death of 125 children due to liver failure caused by consumption of contaminated maize (Aiko and Mehta, 2015). Therefore, the focus on mycotoxins has been a high priority by the FAO and WHO (World Health Organisation), due to their hazardous impacts on human and animal health. This has resulted in strict legislative limits for mycotoxins in many parts of the world in wide range of foodstuffs with the strictest limits in EU (European Commision 2006). In order to facilitate international trade and improve consumer protection, the FAO/WHO organisations have already acnowledged the need to harmonize and standardize mycotoxin maximum limits and sampling plans for all traders of agricultural comoddities in the export market (Magan and Olsen, 2004). Furthemore, a possible climate change effects along with the predicted increase in the population on a global basis, are predicted to have significant impacts on the quality and availability of food crops (Medina et al., 2017). Thus, the capacity to provide additional food of the necessary quality/quantity in the upcoming 50 years has been questioned (Medina et al., 2017). The two most important factors which affect the life cycle of all microorganisms including mycotoxigenic moulds are water availability and temperature (Magan, 2004). The European Food Safety Authority (EFSA) has examined the potential impact of climate change in Europe and has suggested that effects will be detrimental or advantageous depending on geographical region (Battilani et al., 2012; 2016). This suggests that in northern Europe the effects may be positive, while the Mediterranean basin have been identified as a climate change hot spot where extreme changes in temperaure, CO_2 and rainfall patterns are predicted (Magan et al., 2011). This could increase the risk of migration of mycotoxigenic fungi, which might occur as a result of shifts in response to warmer, drought-like climatic conditions (Magan et al., 2011).

Research work has mainly focused on the approx. dozen mycotoxins because of their severe effects on human and animal health and their occurrence in food (Logrieco et al., 2018). Therefore, this research is focused on two of the main contaminants of food and feed, aflatoxin B_1 (AFB_1) and ochratoxin A. Both mycotoxins can be synthesised during the pre-harvest and post-harvest phases (Amezqueta et al., 2012), and are known as a cause of a number of toxic

effects in humans and animals. These mycotoxins account for millions of dollars in losses worldwide annually because of the rejection for food or feed use (Shane, 1994; Vasanthi and Bhat, 1998).

1.1.1 Aflatoxins

Aflatoxins (AF) are amongst the most poisonous mycotoxins and are synthesised by certain moulds (*Aspergillus flavus, A. parasiticus* and *A.nomius*) which grow in soil, decaying vegetation, hay, and grains. Crops that are frequently affected by *Aspergillus* spp. include cereals (corn, sorghum, wheat and rice), oilseeds (soybean, peanut, sunflower and cotton seeds), spices (chili peppers, black pepper, coriander, turmeric and ginger) and tree nuts (pistachio, almond, walnut, coconut and Brazil nut). The toxins can also be found in the milk of animals that are fed with contaminated feed, in the form of aflatoxin M_1 (FAO 1988). Large doses of aflatoxins can lead to acute poisoning (aflatoxicosis) and can be life threatening, usually through damage to the liver (Abrar et al., 2013; Scarpari et al., 2015). The presence of aflatoxins in food/feed can have a variety of toxic effects, e.g., genotoxicity, haemorrhagies, hepatotoxicity, nephrotoxicity, neurotoxicity, oestrogenicity, teratogenicity, immunosuppressive problems, mutagenicity and carcinogenicity (Newberne, 1974; Stark, 1980; Malir et al., 2006; Wild and Gong, 2010; Kensler et al., 2010; Ostry et al., 2016). Aflatoxigenic species produce four types of aflatoxin; B_1, B_2, G_1, and G_2 among which aflatoxin B_1 (AFB_1) is the most hazardous for human and animal health (Ciegler and Bennett, 1980). According to IARC (International Agency for Research on Cancer), AFB_1 is classified as carcinogenic to humans (Group 1A). For this reason, aflatoxins are strictly limited by European laws (EC/1881/2006) (Scarpari et al., 2015).

1.1.2 Ochratoxin A (OTA)

Ochratoxins belong to a group of mycotoxins that are produced as secondary metabolites by fungi, in particular *Aspergillus* and *Penicillium.* The most relevent OTA-producing species among the aforementioned are *Penicillum verrucosum, Aspergillus ochraceus (Aspergillus* section *Circumdati), Aspergillus niger* and *Aspergillus carbonarius* (both belong to *Aspergillus* section *Nigri*) (Amezqueta et al., 2012). Ochratoxins include ochratoxin A (OTA), ochratoxin B (OTB), ochratoxin C (OTC) and ochratoxin α (OT α), of which OTA is considered most toxic (Meulenberg et al., 2012; Malir et al., 2016). According to the FAO, contamination of food commodities, such as cereals and cereal products, coffee beans, cocoa, dry vine fruits, wine and grape juice, spices and liquorice, occurs worldwide. It is known that OTA has nephrotoxic, hepatotoxic, embryotoxic, teratogenic, neurotoxic, immunotoxic, genotoxic and carcinogenic

effect on humans and animals and therefore represents a severe health hazard for humans and animals (WHO 2011) (Van Egmond, 1991). It has also been implicated as a potential causative agent for inducing Balkan Endemic Nephropathy (Pepeljnjak and Šegvić Klarić, 2010). Therefore, the IARC has evaluated the carcinogenic potential of OTA as a possible human carcinogen (Group 2B), based on a large body of evidence of carcinogenicity detected in several animal studies (IARC, 1993).

1.2 Management and regulation of mycotoxins

According to the World Health Organization (WHO), it is almost impossible to produce food completely free from mycotoxins. In particular, AFB_1 and OTA, produced mostly by the *Aspergillus* genus, are amongst the most hazardous mycotoxins, as they are able to contaminate a wide variety of important agricultural products such as peanuts, maize, rice, cottonseed, grape juice, wines and dried wine fruits (El Khoury et al., 2010). Aflatoxins are found in many countries, especially in tropical and subtropical regions, where the conditions of temperature and humidity are optimal for the growth of molds and for the production of mycotoxins. (Rustom, 1996; Sen et al., 2019). The contamination of food and feed by mycotoxins poses major concerns for public health and welfare as the dietary exposure may cause disorders, dysfunctions and alterations of physiological states in both humans and animals (Dellafiora et al., 2017). Moreover, contamination of food and feed with mycotoxins is a worldwide problem. In the recent past, dana on large surveys on mycotoxin occurence across the world have been released suggesting that 25% of world s food crops are contaminated with more than one mycotoxin (FAO) (Schatzmayr et al., 2016). Recent reports, however, indicate that the contamination of commodities (cereals and feed) is much higher: roughly 80 % (Eskola et al., 2019). These figures are expected to rise with the use of more sensitive detection methods and equipment, which will increase the mycotoxin database and standards. Many countries have adopted regulations to reduce the possible dietary intake, thereby preserving the health of animals and consumers (for Europe: EC No 1881/2006, EU No 165/2010, EU No 105/2010). However, the maximum levels of contamination allowed are not harmonized amongst different countries, and this may cause trade frictions at the global level (Dellafiora et al., 2017). The ubiquitous nature of fungi makes food crops vulnerable to mycotoxin contamination both pre-harvest and post-harvest (Hussein and Brasel, 2001). In the field, mycotoxigenic fungi survive on crop debris, as airborne propagules or can be transmitted by insects which can infect ripening crops (Gorman and Kang, 1991; Bottalico, 1998; Cavalluci, 2005). Furthermore, stress

conditions like drought, floods, insect infestation and delayed harvest can increase the level of mycotoxin contamination. Also, post-harvest conditions such as inadequate drying, poor storage environment and management can lead to mould colonisation and toxin contamination (Magan and Olsen, 2004). Thus, the „good agriculture practice„ is essential in minimising mycotoxin contamination.

1.3 Strategies and legislation for controling mycotoxin contamination

Food decay by spoilage fungi causes considerable economic losses and constitutes a health risk for consumers due to the potential for fungi to produce mycotoxins. Thus, efforts continue to be devoted, worldwide, to minimisation, prevention or elimination of mycotoxins (Magan and Olsen, 2004). In the last five decades the major approach for controlling mycotoxin contamination in feed and foodstuffs has been focused on the use of chemicals (Hillocks, 2012). The common use of chemicals such as pesticides, fungistats and fungicides for inhibition of fungal growth and to control mycotoxin synthesis have been shown to have hazardous side effects, including environmental pollution and also allowing the evolution of resistant mycotoxigenic pathogenic strains (Da Cruz Cabral et al., 2013). Consumer awareness of the impacts of such crop protection chemicals on environmental effects and health hazards, has led to a drive to limit their use in agriculture. Since 2014 the European Community has banned about 50% of chemicals used in agriculture (Hillocks, 2012). The Sustainable Use Directive 2009/128/EC states ‘Member states shall take all necessary measures to promote low pesticide-input pest management, giving wherever possible priority to non-chemical methods (European Parliamentary Research Service, 2009). This approach requires creative use of fewer allowable pesticides, integrated with non-chemical methods, to ensure that more compounds are not lost to pesticide resistance and that pests/pathogens are effectively managed (Hillocks, 2012). Integrated Pest Management (IPM) requiers all farms in the EU to conduct their crop protection activities using the principles of IPM, together with the harmonisation of the many environmental schemes (Hillocks, 2012). The combined effects of the EC Pesticides Review, additional legislation to protect water quality and the IPM legislation, means that farmers must make fundamental changes to their farming systems. Designing and incorporating appropriate IPM systems needs to integrate pest management with sustainable farming practices which protect and improve biodiversity and reduce the reliance on synthetic fertilisers, allowing a more eco-friendly farming approach. This approach represents a major challenge to the farmers to produce food commodities without further pressure on the profitability of farming enterprises

and avoiding substantial increases in food prices (Hillocks, 2012). Various novel mycotoxin control strategies in pre-harvest and post-harvest have been developed and are currently being implemented (Varga and Toth, 2005), but none of them represents a permanent solution for mycotoxin control .

1.4 Pre-harvest control

For lowering preharvest contamination, treatment of field crops with fungicides is the traditional technique (Mesterházy and Bartók, 1996). However, environmentally more friendly alternatives have been sought more recently (Varga and Toth, 2005).

1.4.1 Resistance breeding

One of the methods traditionally used to improve the resistance of the host plants to fungal infection is resistance breeding. Enhancing host resistance, has brought some success and a number of resistant/partially resistant lines have been developed, particularly in corn (Barug et al., 2004). Moreover, corn and peanut cultivars resistant to infection by *A. flavus* or aflatoxin accumulation have been developed. Breeding for corn and peanuts have the potential to lower aflatoxin levels in agricultural products (Varga and Toth, 2005). Also, host resistance was found to be a polygenic trait (Tubajika and Damann, 2001). The disadvantages of this process are the long period of time required to produce resistant species, and it is not fully accepted that the designated “resistant” lines are truly resistant. Moreover, these “resistant” genes still have to be incorporated into genotypes with desirable agronomic characteristics for commercial application (Barug et al., 2004). Furthermore this method can lead to the development of resistant species, and therefore is not favored by the scientific community as a means to control the contamination with mycotoxins.

1.4.2 Biological control

One strategy that has shown great promise for reducing mycotoxin contamination in crops involves application of non-toxigenic strains (atoxigenic strains) of the toxigenic species or the same taxonomic group that produces the hazardous toxins (Barug et al., 2004). The use of non-toxigenic strains of *A. flavus* and *A. parasiticus* in biological control effectively reduces aflatoxin contamination in peanuts (Varga and Toth, 2005). Another possibility is the use of

antagonists in the field to control mycotoxin-producing fungi. Moreover, yeasts isolated from almonds and pistachios have also been found to be promising as biocontrol agents against toxigenic *A. flavus* strains (Cleveland et al., 2003).

1.4.3 Genetic engineering

Genetic engineering can either be used to modify plant genes to become less susceptible to fungal infection or mycotoxins, or genes responsible for detoxification can be introduced to the plant. Investigations to inhibit either growth or aflatoxin formation in crops led to the identification of anti-fungal peptide genes which are being used in the genetic engineering of cotton and corn to prevent infection by *A. flavus* (Chen et al., 1998). Expression of anti-fungal peptides in transgenic crops may reduce fungal growth in crop plants and mycotoxin levels in feeds (Varga and Toth, 2005). Transgenes that detoxify mycotoxins or that cause mycotoxins to be eliminated from the plant cells have demonstrated some ability to protect plants against mycotoxins, although concerns on the use of such genetically modified organisms will possibly prevent their widespread use mainly in Europe. Therefore, public and scientific concerns related to the use of genetically modified plants as food or feed ingredients make this approach less suitable than others for mycotoxin control (Varga and Toth, 2005).

1.5 Post-harvest control

Although the prevention of mycotoxin contamination in the field is the main goal of agricultural and food industries, the contamination of various commodities with different myotoxins is unavoidable under certain environmental conditions. For post-harvest control, storage conditions should be improved to minimise the mycotoxin content of foods and feeds (Varga and Toth, 2005). Mycotoxin production is dependent on a number of factors, e.g., water activity of the stored product, temperature, gas composition, the presence of chemical preservatives and microbial interactions. An integrated approach for controlling several of these factors could give much more effective control of deterioration without requiring extreme control of any one factor. Decontamination/detoxification procedures are useful in order to recuperate mycotoxin contaminated commodities.

Several strategies are available for the detoxification or decontamination of commodities containing mycotoxins. These can be classified as physical, chemical and (micro)biological approaches.

1.5.1 Physical methods

Mechanical separation, density segregation, colour sorting, removal of the fines or screenings from the bulk shipments of grains and nuts are able to significantly reduce the mycotoxin content of grains. Gamma irradiation has successfully been used to control ochratoxin A levels in feeds (Refai et al., 1996). In some cases, if the mycotoxin levels are known, it is also possible to dilute out the effects of certain contaminated raw materials by blending to produce a final blended feed below the critical level of the specific mycotoxin (Schaafsma, 2002). Notably, according to EU laws (EC 401/2006 and EC 1881/2006) dilution is not alowed for food contaminated by mycotoxins, or if the feed was contaminated by aflatoxins.

1.5.2 Physicochemical methods

The utilisation of mycotoxin-binding adsorbents is the most widely applied method for protecting animals against the harmful effects of mycotoxin-contaminated feed. The efficiency of the adsorption depends on the chemical structure of both the adsorbent and the mycotoxin (Varga and Toth, 2005). Polarity of electrical charges on the surface of adsorbent particles, pore size and pH of the adsorbents also affect mycotoxin binding (Perkins, 1999). Furthermore, aflatoxins have a strong positive charge, therefore they can be adsorbed by polar adsorbents such as hydrated sodium calcium aluminosilicates (HSCAS)(Phillips et al., 1988). Although, the efficacy of HSCAS was quite limited against zearalenone and ochratoxin A (Huwig et al., 2001). Beneficial effects of activated carbon have been shown in rats intoxicated with T-2 toxin, and in vitro activated carbon showed the capacity to adsorb fumonisin B1 and ochratoxin A from aqueous solutions (Huwig et al., 2001; Varga and Toth, 2005). The application of adsorbents for reducing the toxic effects for livestock is widespread. However, not a single adsorbent has been shown to be effective against most types of mycotoxins. Besides, several adsorbents which were found to be effective in *in vitro* tests failed to do so in *in vivo* experiments (Varga and Toth, 2005).

1.5.3 Chemical methods

A wide variety of chemicals have been found to be effective (to different extents) against mycotoxins. The chemicals used fall into the categories of aliphatic acids and their salts (calcium prionate, potassium sorbate), bases (e.g., ammonia, sodium hydroxide), oxidising reagents (e.g., hydrogen peroxide, ozone), reducing agents (e.g., bisulphite, sugars), chlorinating agents (e.g. chlorine), salts and miscellaneous reagents such as formaldehyde.

Ammoniation is the method that has received the greatest attention for detoxification of aflatoxin or ochratoxin-contaminated feeds and has been used successfully in several countries (Chelkowski et al., 1982; Varga and Toth, 2005). Sodium bisulphite has been shown to react with aflatoxins and trichothecenes to form sulphonate derivatives while peroxide and heat enhance the destruction of AFB_1 by sodium bisulphite. Destruction of OTA has been observed after treatment with sodium hypochlorite (Varga and Toth, 2005). Ozone treatment has also been used successfully for decontamination of food products (McKenzie et al., 1997). Chemical methods can be used effectively to reduce mycotoxin levels in food and feeds. However, these chemicals can cause changes in the nutritional properties of food and feed, and also could cause environmental problems, rendering this approach less favourable.

1.5.4 Biological degradation methods

The history of biocontrol applied to molds and mycotoxins began in the 1960s, when Ciegler et al. (1966) screened over 1000 microorganisms for their ability to degrade aflatoxins, and identified *Flavobacterium aurantiacum* as capable of irreversibly removing aflatoxin from solutions. In later studies, it was observed that the activity of *F. aurantiacum* was influenced by pH and temperature (Lillehoj et al., 1967). In the literature, mold growth has been described as controlled by other filamentous molds, yeasts and bacteria (Bianchini and Bullerman, 2009). Biological detoxification can be defined as the biotransformation of mycotoxins or enzymatic degradation that can be obtained by either the whole cell or an enzyme system (Bata, 1999). *F. aurantiacum* is able to irreversibly remove AFB_1 from both solid and liquid media (Lillehoj et al., 1967). The ability of this microorganism to remove aflatoxins from foods was demonstrated in various food commodities including vegetable oil, peanut, corn, peanut butter and peanut milk. Recent studies indicated that the factor responsible for degradation of AFB_1 by the extract of *F. aurantiacum* is an enzyme (Smiley and Draughon, 2000). The possibility of using the purified crude protein extracts in the food industry was suggested as a means of removing aflatoxins from contaminated foods. Liu et al. (2001) isolated and characterised the enzyme responsible for aflatoxin degradation (the so-called aflatoxin-detoxifizyme) from *Armillariella tabescens.* This multienzyme complex is possibly responsible for opening the difuran ring of AFB_1, thus leading to decreased mutagenic activity (Liu et al., 2001).

Furthermore there have been several microorganisms such as *Aspergillus niger, A. fumigatus*, *Lactobacillus*, *Streptococcus*, *Bifidobacterium* and *Rhizopus* spp. which have been screened for their ability to degrade OTA (Skrinjar et al., 1996; Varga et al., 2000; Abrunhosa et al., 2002).

These microorganisms are able to convert OTA to the far less toxic ochratoxin α. However, the pathway leading to the opening of the isocoumarin ring is unknown (Varga et al., 2000). Also, the authors have demonstrated that *A. niger* strain CBS 120.49 could effectively eliminate OTA both from liquid and solid media, and the degradation product, ochratoxin α was also decomposed. This atoxigenic *A. niger* strain, or its enzymes responsible for OTA degradation, for its detoxification in agricultural products with low water activities such as cereal grains and green coffee beans (Varga and Toth, 2005). Moreover, *Rhizopus* isolates were able to degrade OTA only partially within 10 days (Varga and Toth, 2005). The enzyme involved in the reaction is possibly a carboxypeptidase, as carboxypeptidase A can convert ochratoxin A to ochratoxin α (Deberghes et al., 1995; Stander et al., 2001). Although biosynthetic genes of several mycotoxin-degrading enzymes have been cloned and characterised, none of them are used routinely for mycotoxin control in foods or feeds (Varga and Toth, 2005). Lactic acid bacteria, *Bacillus* species have also been reported as capable of binding mycotoxins, which shows promise for the possible use of these organisms as sequestering agents in fermented and other processed foods, as well as in the gut (Bianchini and Bullerman, 2009). Gourama & Bullerman (1995) have reported that effect of small molecular weight metabolites produced by *Lactobacillus* species inhibits the aflatoxin production after 14 days of incubation. In the literature there is also evidence of microbial degradation of mycotoxins, and while this area of research is promising, there are some risks associated with potential production of toxic by-products, only partial detoxification, or masking/modification of mycotoxins that have the same toxicity during digestion (Kovač et al., 2018) that need further study.

1.5.5 Plant extracts as as tool for mycotoxin inhibition

Numerous studies have demonstrated that plant extracts contain diverse bioactive components that can control mould growth and mycotoxin production. The metabolites produced by plants are a promising alternative because plants generate a wide variety of compounds, either as part of their development or in response to stress or pathogen attack. They are easily decomposed, environmentally friendly and non-phytotoxic (Da Cruz Cabral et al., 2013). It has been proven that plant extracts obtained with different solvents and essential oils have antimicrobial activity for plant protection, including alkaloids, flavonoids, isoflavonoids, tannins, cumarins, glycosides, terpenes, phenylpropannes, and organic acids (Da Cruz Cabral et al., 2013). Kumar et al. (2007) demonstrated that *Chenopodium ambrosioides* oil inhibited the mycelial growth of two aflatoxigenic strains of *Aspergillus flavus*. This oil also inhibited the growth of

Aspergillus fumigatus, Botryodiplodia theobromae, Fusarium oxysporum, Phythium debaryanum and *Sclerotium rolfsii*. Dos Santos Oliveira and Badiale Furlong, (2008) showed that phenolic compounds contained in methanolic extracts from banana pulp and peel; orange, eggplant and potato pulp inhibits aflatoxin synthesis in *A. flavus*. Moreover, essential oils containing α-terpinolene, α-terperpine, eugenol, α-cariofilen extracted from *P. boldus* and *S. aromaticum* showed significant reduction in OTA synthesis in species from the *Aspergillus* section *Nigri* (Passone et al., 2012). Also extracts from marine organisms have shown antifungal potential against main producers of mycotoxins (*A. flavus, A. ochraceus, F. graminearum, F. verticillioides*) (Jerković et al., 2019). At present, extraction of bioactive compounds requires the utilization of large amounts of solvent and technologies that are not environmentally friendly. Therefore, further systematic studies are needed to broaden the knowledge in this area.

1.6 Oxidative stress as a „ prerequisiste“ for aflatoxin and ochratoxin A production

Oxygen is essential for the life of aerobic organisms, but due to its ability to inactivate enzymes and because it causes formation of reactive oxygen species (ROS), when supplied at concentrations only slightly greater than those in normal air, the oxygen may cause toxic effects in aerobic organisms (Halliwell, 1978). Aerobic cells have developed a mechanism of production to cope with the harmful effects of oxygen in the environment by formation of (ROS) such as superoxide anions, hydroxyl radicals, singlet oxygen, hydrogen peroxide or oxylipins from unsaturated fatty acid. These reactive compounds are formed during metabolic processes, such as glucose respiration and fatty acid metabolism, by the activity of NADPH oxidases and other oxygenases (Reverberi et al., 2010). Moreover, ROS can be overproduced following the action of oxidative stressors present in the environment (Kappus, 1985; Passi et al., 2005; Reverberi et al., 2001, 2005; Halliwell and Gutteridge, 2007). These molecules have very reactive properties and imbalance between oxidants and antioxidants inside the cell can cause a state of an “oxidative stress“ which leads to probable damage of the cell constitutes (Sies, 1991). In general, the cell can tolerate a certain amount of ROS by increasing the synthesis of antioxidant molecules (e.g., α-tocopherols, ascorbic acid, carotene, reduced glutathione), and by increasing primary and secondary enzymatic systems to avoid ROS-induced damage (Passi et al., 2005). Enzymatic systems include formation of enzymes such as superoxide dismutase (SOD), catalase (CAT), and glutathione peroxidase (GPX) (da Silva et

al., 2018). The enzymes are characterised as primary antioxidant enzymes that trigger the breakdown of ROS (da Silva et al., 2018). The mechanisms of action of these enzymes are diverse. SOD breaks down the superoxide anion radical into H_2O and O_2, whereas CAT catalyses the decomposition of hydrogen peroxide (H_2O_2) into water and oxygen. GPX reduces hydrogen peroxide to water (Reverberi et al., 2008). The synthesis of mycotoxins by fungi is genetically determined and closely related to primary metabolic pathways, such a s amino acid and fatty acid metabolism. However, the mycotoxin production is also modulated by environmental factors such as substrate composition and quality, humidity and temperature (Makun, 2013). It is known from the literature that intracellular oxidative stress can be considered as a „ trigger“ for mycotoxin production (Reverberi et al., 2001, 2005, 2008, 2009; Roze et al., 2011). Reverberi et al. (2009) and Fanelli (2004) demonstrated that oxidative stress and AFs synthesis are very closely related events. Morevoer, studies *in vitro* (Jayashreea and Subramanyam, 2000) and *in vivo* (Abdel-Wahhab and Aly, 2003; Fanelli et al., 2004) demonstrated that oxidative stress plays a major role in the toxic effects of AFB_1. The main consequences of ROS generation induced by AFB_1 are damage to DNA (Wang et al., 2017; Zhang et al., 2016). Recent studies have shown that increase of the concentration of antioxidant mechanisms such as SOD, GPX and CAT expression within the cell can lead to blockade of AFB_1 synthesis (Reverberi et al., 2005; Liu et al., 2017). Furthermore, Reverberi et al. (2008, 2010) demonstrated that the synthesis of both AFB_1 and OTA are genetically regulated. The fungal cells cointain oxidative stress-related receptors that can transduce the „oxidative message“ to the nucleus and promote a transcriptional change targeted at restoring correct redox balance in the cell. Ultimately, the authors demonstrated that in *A. parasiticus* the Apyap1 gene is implicated in the modulation of aflatoxin biosynthesis following the perturbation of redox balance (Reverberi et al., 2008). Furthermore, in *A. ochraceus*, the Aoyap1 gene contributes to control the redox balance in the cell, which can lead to inhibition of ochratoxin A synthesis (Rodrigues–Pousada et al., 2010). Due to antioxidants ability to regulate the oxidant/antioxidant balance in the cell, various studies have been conducted on the effect of antioxidants in inhibiting the control of AFB_1 and OTA synthesis (Fanelli et al., 1985; Kim et al., 2004). One of the promising tools for mycotoxin control (particularly aflatoxins and OTA), with lower environmental impact, are the metabolites of higher mushrooms.

1.7 Mushroom polysaccharides

Mushrooms have unique sensory properties and nutritional values as well as health benefits due to their bioactive compounds, especially β-glucans. They have a long history in culinary and medicinal use (Sari et al., 2016). Edible mushrooms are regarded as healthy food and nutrient sources because of their many beneficial components, including carbohydrates, dietary fiber, protein, vitamins and minerals, and low levels of calories, fat and toxic metals (Zhang et al., 2016). Moreover, human beings have used mushrooms as medicines for 5000 years or more (Halpern, 2010). The most cultivated mushroom species are *Agaricus bisporus*, *Lentinula edodes*, *Pleurotus* spp., *Trametes versicolor* and *Shizophyllum commune*, and their production is continuously increasing in China, which is the largest producer worldwide (Aida et al., 2009; Patel and Goyal, 2011). Due to their unique sensory properties, nutritional values and health benefits, mushrooms have become the focus of international medicinal research in recent years. The dry matter content is very low in mushrooms (approximately 10%). Furthermore, high insoluble fibre contents (chitin and other polysaccharides) present nutritional advantages, and low lipids and glycogen contents result in low energy values (Kalac, 2009, 2012). In addition to a wide variety of compounds that are beneficial to health, such as phenolic substances, sterols, alkaloids, lactones, terpenes and ceramides, bioactive polysaccharides and polysaccharide-protein complexes are the most studied group of functional compounds in medicinal mushrooms (Hishida et al., 1988; Villares et al., 2012; De Silva et al., 2013). These glucans show bioactive properties such as immune-modulating, antitumor (Sari et al., 2016), antiviral (Zhang et al., 2007) and hepato-protective effects (Wasser, 2014). The major structural feature of mushroom beta-glucans is a β-1,3-D-glucan main chain with single D-glucosyl residues linked β-1,3 along this main chain. Some of this glucan can be extracted from the fruiting body of the mushroom, and soluble β-glucans are also produced by cultured mycelia (Chang and Wasser, 2012). Because β-glucans are not synthesized by the human body, they are recognized by the immune system and induce both adaptive and innate immune responses (Brown and Gordon, 2005). In this context, the use of mushroom extracts with soluble β-glucans vs. the consumption of the whole fruiting body is discussed for digestibility and bioactivity (Kalac, 2012; Wasser, 2014). Furthermore, chitin and α-glucans are present in mushrooms; the total polysaccharide contents of mushrooms range between 50% and 90% (Wasser, 2002).

1.7.1 Mushrooms as biological response modifiers

Excess production of free radicals can cause damage to DNA, lipids and proteins, which in turn can result in several chronic diseases, such as cardiovascular diseases, cancer and neurodegenerative diseases (Sari et al., 2016). Mushrooms contain large amounts of mycochemicals that possess antioxidant properties and have a strong free radical scavenging ability (Islam et al., 2019). Many, if not all, higher basidiomycetes mushrooms contain biologically active polysaccharides in fruit bodies, cultured mycelium, and cultured broth (Kobayashi et al., 1993; Sun et al., 2004, Zjalić et al., 2006), and they are generally considered to be the main contributors to the antioxidant activity (Zhang et al., 2016). Some of these poysaccharides are described as biological response modifiers (BRM); these include compounds with a specific biological function: antibiotics (e.g., plectasin), immune system stimulator (e.g., lentinan), antitumor agents (e.g., krestin, PSK) and hypolipidemic agents (e.g., lovastatin), inter alia (Zhang et al., 2016; Scarpari et al., 2015). Mushrooms exo-polysaccharides demonstrated high results in antioxidant activity, including inhibition of lipid peroxidation, reduction of human low-density lipoproteins. Furthermore they have the ability of scavenging of free radicals, which enables polysaccharides to act as biological response modifiers (BRM).

1.8 Mushroom polysaccharides as a possible tool for mycotoxin control

The possibility of using mushroom polysaccharides as a means to control AFB_1 synthesis has been widely demonstrated (Ricelli et al., 2002; Zjalić et al., 2006; Scarpari et al., 2014, 2017; Khatoon and Abidin, 2018). Scientific studies on more than 99 polysaccharides of different mushrooms and their impact on mycotoxin synthesis have been ongoing for more than 20 years, due to their high efficiency in controling the mycotoxin synthesis. The studies have been focused on specific isolates of the two lygnolitic mushrooms: *Lentilula edodes* and *Trametes versicolor* (Zjalić et al., 2006). Lyophilised culture filtrate from *L. edodes* have shown a stimulating effect on the activation of transcription factors related to anti-oxidant response and anti-oxidant enzyme activity with a contemporaneous delay in aflatoxin genes transcription, which led to a marked reduction of AFB_1 production (Reverberi et al., 2005). Moreover, *L. edodes* polysaccharides have lignolytic properties that can contribute to the degradation of substances such as olive oil mill waste waters, which are byproducts of olive oil industries and represent a pollution problem in all Mediterranean countries (Zjalić et al., 2006). Furthermore, the mushroom *T. versicolor* produces and secretes the exopolysaccharides and glycoproteins in

culture filtrate, which are responsible for the modulation of several biological responses (Wasser and Weiss, 1999). It has been demonstrated that polysaccharide from mushroom *T. versicolor* shows the long-lasting ability to inhibit the synthesis of mycotoxins in *A. flavus*, *A. parasiticus* and *A. carbonarius* both in vitro and in vivo (Zjalic et al., 2006; Scarpari et al., 2014). Some of the major factors that affecting mycotoxin biosynthesis in a critical and pivotal role is played by intracellular and environmental oxidative stress. The close relationship between endogenous oxidative state and aflatoxin biosynthesis and the direct proportion between increased levels of both reactive oxygen species (ROS) and aflatoxin biosynthesis in *A. parasiticus* has been demonstrated (Reverberi et al., 2008, 2010; Christensen & Kolomiets, 2011; Scarpari et al., 2017).

1.8.1 *Trametes versicolor* polysaccharides as a possible tool for mycotoxin control

The background of this research is based on the study of the Chinese medicinal mushroom "yun zhi", *T. versicolor*, traditionally used for "replenish essence and qi (vital energy)" (Zjalić et al., 2006). Previous studies indicated the potential activity of extracts from culture filtrate of asexual mycelia of *T. versicolor* in controlling the growth and secondary metabolism (e.g., mycotoxins) of plant pathogenic fungi. Scarpari et al. (2015) have demonstrated that semi-purified culture filtrates of *T. versicolor* containing molecules with a supposed molecular mass (MM) of >3.0 kDa showed a particular ability in enhancing antioxidant activity in *A. flavus*, dramatically inhibiting the biosynthesis of aflatoxins. In relation to this, the ability of the semi-purified culture filtrate of *T. versicolor* to inhibit aflatoxin biosynthesis was further exploited. Scarpari et al. (2017) were able to identify the active principles produced by *T. versicolor*, which was an exo-polysaccharide released in its culture filtrate, called Tramesan. Tramesan has a biological activity in blocking AFB_1 and OTA production. Biological active saccharidic fraction in the filtrate of the medicinal mushroom *T. versicolor* has been partial chemicaly characterizatised. It has been shown to have the ability to trigger an antioxidant response in different biological systems (human cell lines, plants, and fungi) (Ricciardi et al., 2017). The active component of the extracts were found to be a polysaccharide. The polysaccharide was isolated, part of its primary structure determined and registred as Tramesan. Tramesan is a branched fucose-enriched fungal polysaccharide of about 23 kDa with a probable "repetitive" scheme of monosaccharide sequence in the linear $(\alpha\text{-}1,6\text{-}Gal)_n$ backbone as well as in the lateral chain Man-(1→2)-Man-(1→3)-Fuc (Scarpari et al., 2017). It is able to act as a pro-antioxidant in different organisms. By enhancing the "natural" antioxidant defences of the "host", Tramesan

could represent a useful tool for controlling the synthesis of several mycotoxins (Zjalic et al., 2006; Reverberi et al., 2005). As demonstrated by Reverberi et al. (2010), Tramesan is able to elicit an antioxidant response, probably by acting on gene expression. These authors suggested that Tramesan could be recognized by specific receptors that, in turn, activate pathways leading to an antioxidant response. Furthermore, Tramesan could act as ligand for a still unknown inter-kingdom conserved receptor able to control antioxidant responses. All the above results suggested that Tramesan could represent a useful tool for different challenges in different "contexts", in which, limiting ROS production represents a solution for minimising the release of toxins in foodstuffs. Even if Tramesan is a very promising tool in the challenge against the presence of mycotoxins in food and feed, there are some possible problems in its application. The production costs could be too high to be suitable for industrial use. Moreover, Tramesan is produced by a living organism and some mutation(s) could alter its structure and efficacy. A solution could be to define the structure of the most active fragment of the polysaccaride and synthesize it in the laboratory. To proceed in this direction, a further investigation on the structure of oligomers obtained from Tramesan was carried out in the present thesis.

1.8.2. Oligosaccharides derived from Tramesan: their structure and activity on mycotoxin inhibition in *Aspergillus flavus* and *Aspergillus carbonarius*

In order to evaluate the smallest component active in AFB_1 and OTA inhibition, the polysaccharide was hydrolyzed into fractions from disaccharides to heptasaccharides. The fractions of different lengths were separated and tested for their mycotoxin inhibition activity.

1.8.3 Wide screening of mushroom polysaccharides and their effect on mycotoxin inhibition in *Aspergillus flavus* and *Aspergillus carbonarius*

In order to determine the exact active component of the polysaccharide structure that was most effective in inhibiting mycotoxin synthesis, and where this was similar to the actions of Tramesan, a wide range of mushroom strains were screened. The goal was to identify the tool which could be effectively used for the significant control of AFB_1 and OTA production, for use in food and feed applications. Furthermore, the aim was the clarify the structure of the mushroom polysaccharides capable of inhibiting the synthesis of mycotoxins, so that it could be established whether there is a common structure that can be used in designing synthetic glucans for mycotoxin control. In this study, three different variants of polysaccharide mushroom *Schizophyllum commune*, including commercial Schizophyllan, semi-purified exo-

polysaccharide from *S. commune* and non-purified culture filtrate of *S. commune* polysaccharides were assayed for inhibition of mycotoxin synthesis in *A. flavus* and *A. carbonarius*. In addition, some studies were carried out on their effects on the antioxidant responce in fungal cells.

The goals of this project were:

1) Examine the structure of Tramesan, to elucidate the minimal portion of the polysaccharide (oligosaccharide) efficient in blocking mycotoxin biosynthesis for the purpose of its synthetic manufacture (Chapter 2).

2) Comparison of the structures of different mushrooms exo-polysaccharides active in mycotoxin control, to establish whether there is a common structure efficient in exploiting mycotoxin inhibition similar to Tramesan. This would enable economic production of this biocompatible tool for simultaneous inhibition of mycotoxins (Chapter 3).

3) Elucidation the the possible common mechanisms of action between lyophilized raw extract of different mushrooms and Tramesan, on antioxidant activity in mycotoxigenic fungi (Chapter 4).

1.9 References

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Chapter 2. Oligosaccharides derived from Tramesan: their structure and activity on mycotoxin inhibition in *Aspergillus flavus* and *Aspergillus carbonarius*

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Abstract

Food and feed safety, due to its direct influence on human health, has acquired a paramount relevance in everyday life. The awareness that different chemicals, e.g., those largely used in agriculture to control pests, could present both environmental problems and health hazards, has led to a large limitation of their use. Since 2014, the European Community (EC) has banned about 50% of crop protection chemicals used in agriculture. In plant cultivation, chemicals were also the main tool used against fungal pathogens and against the contamination of food/feed stuff with toxic secondary metabolites, mycotoxins. There is thus a drive to develop more environmentally friendly "green" approaches and eco-compatible tools to control mycotoxigenic fungi and toxin contamination of staple foodstuffs. Different mushroom metabolites showed the potential to act as control agents against fungal growth and mycotoxin production. This study has examined the use of a polysaccharide Tramesan, extracted from the basidiomycete *Trametes versicolor* for controlling mycotoxin biosynthesis by *Aspergillus flavus* (aflatoxin B1) and *Aspergillus carbonarius* (ochratoxin A). This study evaluated component of the repeating unit of the polysaccharide produced by *T. versicolor* which was active in inhibition of aflatoxin B1 synthesis. The purified exopolysaccharide of *T. versicolor* was partially hydrolyzed and separated by column chromatography into fractions from disaccharides to heptasaccharides. Each fraction was individually tested for mycotoxin

inhibition in *A. flavus* and *A. carbonarius*. Although fragments smaller than seven units showed no significant effect on mycotoxin inhibition, heptasaccharides showed inhibitory activity of up to 90% in both fungi. These results indicated that these compounds could be used as natural alternatives to crop protection chemicals for control of these two mycotoxins in economically important commodities.

2.1 Introduction

Mycotoxins are fungal secondary metabolites that represent a serious threat to both animal and human health (Reverberi et al., 2010). The contamination of food and feed by mycotoxins poses major concerns for the public health and welfare as the dietary exposure may cause disorders, dysfunctions and alterations of physiological states in both humans and animals (Dellafiora et al., 2017). Ripening staple crops are all exposed to phyllosphere fungi, some of which are able to infect the crops and result in mycotoxin contamination. Many countries have strict regulations to minimise the exposure of humans and animals to the key mycotoxins (Europe: EC No 1881/2006, EU No 165/2010, EU No 105/2010). However, the allowed levels of contamination are not harmonized among countries, and this may cause trade frictions at the global level (Dellafiora et al., 2017). The presence of aflatoxins (AFs) in food and feed can have a variety of toxic effects, e.g. haemorrhagies, hepatotoxicity, nephrotoxicity, neurotoxicity, oestrogenicity, teratogenicity, immunosuppressive problems, mutagenicity and carcinogenicity (Newberne, 1974; Stark, 1980; Malir et al., 2006; Wild and Gong, 2010; Kensler et al., 2011; Ostry et al., 2017). It is known from the literature that aflatoxins are capable of inducing liver tumor and immune-depression in humans and animals (Abrar et al., 2013; Scarpari et al., 2016). According to IARC (International Agency for Research on Cancer) aflatoxins are classified as carcinogenic to humans (Group 1A). For this reason, aflatoxins are strictly limited by European laws (EC/1881/2006) (Scarpari et al., 2016). Moreover, Ochratoxin A (OTA) represents a severe health hazard for humans and animals (WHO, 2011). OTA contamination occurs in a variety of food and feed such as coffee beans, spices, meat, cheese product and wine (El Khoury et al., 2010). Moreover, studies have shown that OTA has nephrotoxic, hepatotoxic, embryotoxic, teratogenic, neurotoxic, immunotoxic, genotoxic and carcinogenic effect on humans and animals (Van Egmond, 1991). Therefore, the IARC evaluated the carcinogenic potential of OTA as a possible human carcinogen (Group 2B), based on a large body of evidence of carcinogenicity detected in several animal studies (WHO-IARC, 1993a, b). The common use of chemicals (pesticides and fungicides) for inhibition of fungal

growth and to control mycotoxin synthesis have shown hazardous side effects, such as strong environmental pollution with severe damage for the human and animal health and selection of resistant strains. The raising awareness that different chemicals could cause both environmental problems and health hazard, led to large limitation of their use in agriculture. The European Community (EC) has banned about 50% of the chemicals commonly used in crop production since 2014 (EC/129/2009; Scarpari et al., 2016). In addition, today, the EC policy is driving research to investigate more environmentally friendly "green" approaches. This has increased the research on the development of biocontrol strategies as well as the use of natural plant extracts to control and mitigate the presence of mycotoxins in food and feed (Lakshmeesha et al., 2019; Degola et al., 2019; Gómez et al., 2018; Elhouiti et al., 2017; Parroni et al., 2019). One of the promising tools for mycotoxin (particularly aflatoxins) control, with lower environmental impact, are the metabolites of higher mushrooms. It has been demonstrated that among the factors affecting mycotoxin biosynthesis in *A. flavus* and *A. parasiticus*, a critical and pivotal role is played by intracellular and environmental oxidative stress. The close relationship between endogenous oxidative state and AF biosynthesis and the direct proportion between increased levels of both reactive oxygen species (ROS) and aflatoxin biosynthesis in *A. parasiticus* has been demonstrated (Reverberi et al., 2008, 2010; Christensen & Kolomiets, 2011; Scarpari et al., 2016). Mushrooms contain large amounts of mycochemicals that possess antioxidant properties and have a strong free radical scavenging ability (Islam et al., 2019). Many, if not all, higher basidiomycete mushrooms contain biologically active polysaccharides in fruit bodies, cultured mycelium, and cultured broth (Kobayashi et al., 1993; Sun et al., 2004; Zjalić et al., 2006), and they are generally considered to be the main contributors to the antioxidant activity (Zhang et al., 2016). Some of these poysaccharides are described as biological response modifiers (BRM); these include compounds with a specific biological functions: antibiotics (e.g. plectasin), immune system stimulator (e.g. lentinan), antitumor agents (e.g. krestin, PSK) and hypolipidemic agents (e.g. lovastatin), inter alia (Zhang et al., 2016; Scarpari et al., 2017). The possibility of using mushroom polysaccharides as a means to control aflatoxins synthesis has been widely demonstrated (Ricelli et al., 2002; Zjalić et al., 2006; Reverberi et al., 2005, 2011; Scarpari et al., 2014, 2016; Khatoon et al., 2018). Probably, the most studied are the polysaccharides produced by the mushroom *Trametes versicolor*. It has been demonstrated that *T. versicolor* lyophilised culture media and mycelial extracts showed long-lasting ability to inhibit the synthesis of aflatoxins both *in vitro* and *in vivo* (Zjalic et al., 2006; Scarpari et al., 2014; Reverberi et al., 2007). The active component of the extracts was found to be a polysaccharide. The polysaccharide was isolated, part of its primary structure

determined and registred as Tramesan. Tramesan is a branched fucose-enriched fungal polysaccharide of about 23 kDa with a probable "repetitive" scheme of monosaccharide sequence in the linear $(\alpha\text{-}1,6\text{-}Gal)_n$ backbone as well as in the lateral chain Man-(1→2)-Man-(1→3)-Fuc (Scarpari, et al., 2017). Tramesan is able to act as a pro antioxidant in different organisms. By enhancing the "natural" antioxidant defences of the "hosts", Tramesan could represent a useful tool for controlling the synthesis of several mycotoxins simultaneously (Ricelli, et al., 2002; Reverberi, et al., 2005; 2011, Zjalic, et al., 2006; Scarpari, et. al., 2016, 2017). As demonstrated by Reverberi et al. (2010), Tramesan is able to elicit an antioxidant response, probably by acting on gene expression. They suggested that Tramesan could be recognized by specific receptors that, in turn, activate pathways leading to an antioxidant response. Tramesan could act as ligand for a still unknown inter-kingdom conserved receptor able to control antioxidant responses. This suggests that Tramesan could represent a useful tool for minimising toxin impacts in food and feed. There is little information on the components of the Tramesan extract which are responsible for control of mycotoxin production.

The objectives of this study were to evaluated the smallest active component of Tramesan for aflatoxin B1 and ochratoxin A inhibition. Tramesan was hydrolyzed and fractions were separated and individually tested for their mycotoxin inhibition activity, with a focus on AFB_1 and OTA control.

2.2 Materials and methods

2.2.1 Fungal strain and growth conditions

T. versicolor used in this study was registered at CABI Biosciences (UK) and deposited in the culture collection of Department of Environmental Biology of Sapienza University of Rome as ITEM 117. *T. versicolor* strain 117 was grown for 7 days in potato dextrose broth (PDB, HiMedia, India) and incubated at 25˚C under shaken conditions (100 rpm). The liquid culture was homogenized, in sterile conditions, in Waring blender 8012. After homogenization, an aliquot (5% v/v) of the fungal culture was inoculated in sterile conditions in 500 mL of PDB in 1L-Erlenmeyer flasks and incubated for 14 days at 25˚C under rotary shaken conditions (100 rpm). The fungal biomass was then separated from the culture filtrates by subsequent filtration with different size filters (Whatman), to eliminate all the mycelia. Mycelia-purified culture filtrate was evaporated under reduced pressure by rotary evaporation (IKA, RV 10, basic). 1L of culture filtrate was concentrated to a volume of 50 mL, lyophilized and utilized for

subsequent analyses. The isolates were kept on Potato Dextrose Agar (Himedia) at 4˚C and the cultures were sub-cultured every 30 days.

2.2.2 Purification of polysaccharide fractions

Purification of *Trametes versicolor* was performed as described in Scarpari et al., (2017). The lyophilized *T. versicolor* culture filtrate (1g) was dissolved in 30mL of ultrapure H_2O and filtered to separate the insoluble components from the soluble ones. The solution was cooled at 4°C and precipitated with 4 volumes of cold absolute EtOH. The precipitate was recovered by centrifugation at 4000 rpm for 20 min at 4°C. The recovered pellet was re-suspended in 4 mL of ultrapure H_2O and 4 mL of 20 mM phosphate buffer pH 7.5 to achieve the optimal conditions of ionic strength and pH for pronase E (Sigma-Aldrich) activity. The proteolysis was carried out at 37°C overnight. The sample was centrifuged (4000 rpm/ 40 min at 4°C) and the supernatant was collected, dialysed (10 kDa membrane cut off) and recovered by lyophilisation (yield~60 mg/L).

2.2.3 Tramesan oligosaccharide production and characterization

After deacetylation with 0.1 M NaOH at room temperature for 5h, under N_2 flow, the polysaccharide was subjected to low pressure size exclusion chromatography (SEC) on a Sephacryl S-300 column to separate the proper molecular mass range and further purify the polymer from contaminants (fractionation domain: 1–400 kDa for dextrans; gel bed volume: 1.6 id x 90 cm), using 50 mM NaNO3 as eluent at a flow rate of 6 mL/h. The gel performances of the column allowed the discrimination of the polysaccharides with a molecular weight between 2 and 400kDa. The sample was separated in three loads; 1 = 50 mg, 2 = 43,1 mg, 3 =40 mg. For the first loading the sample was dissolved in 1.9 mL of eluent and centrifuged 10 min at 13000 rpm, and subsequently it was loaded in a column. Fractions of 2 mL were collected at 20 min intervals. The same procedure was repeated with the remaining 2 parts of the sample. Elution was monitored using a refractive index detector (WGE Dr. Bures, LabService Analytica), connected to a paper recorder and interfaced with a computer via PicoLog software.

The polysaccharide fractions obtained from SEC were subjected to mild acid treatment in order to produce oligosaccharides suitable for low pressure size exclusion chromatography using the Biogel P2 (column range 100-1800 Da). The partial hydrolysis reaction was performed by dissolving the polymer in water until a concentration of 2 mg/mL. After heating for

15min/100°C, 2M triflouracetic acid was added and the sample incubated for 2h/100°C. The fractions were separated by size exclusion chromatography on a Bio Gel P2 column (fractionation domain: 100–1800 Da, gel bed volume 1.6 cm i.d. X 90 cm), using 50 mM $NaNO_3$ as eluent at flow rate of 6.4 mL/h. Fractions of 1.5 mL were collected at 15 min intervals and those belonging to the same peak were pooled together and desalted on a Bioline preparative chromatographic system equipped with a Superdex G30 column, previously equilibrated in H_2O. Fraction (B1+B2) showed five different peaks (B1, B2, B3, B4, B5). Elution of each peak was monitored with a Refractive Index detector (Knauer, LabService Analytica). The purified and desalted fractions were liophylised and prepared for NMR and ESI-MS analysis.

2.2.4 NMR spectroscopy

The purified oligosaccharides were analysed by NMR spectroscopy. The samples were exchanged two times with 99.9% D_2O by lyophilisation and then dissolved in 0.6 mL of 99.96% D_2O. Spectra were recorded on a 500 MHz VARIAN spectrometer operating at 25˚C for oligosaccharide solution. 2D experiments were performed using standard VARIAN pulse sequences and pulsed field gradients for coherence selection when appropriate. Standard parameters were used for 2D NMR experiments. Chemical shifts are expressed in ppm using acetone as internal reference (2.225 ppm for ^{1}H and 31.07 ppm for ^{13}C). NMR spectra were processed using MestreNova software.

2.2.5 Inhibition of aflatoxin B_1 and OTA biosynthesis in *A. flavus* 3357 and *A. carbonarius* by *T. versicolor* oligasaccharide fractions

A. flavus (Speare) NRRL 3357, a high AFB_1 producer and *A. carbonarius* producer of OTA were grown on PDA (HIMEDIA) at 30˚C for 7 days in dark conditions. 90 µL of double concentrated potato dextrose broth (48 g/L) (HIMEDIA), 100 µL of mixture of the different oligosaccharides in sterile water), and 10 µL of conidial suspension in sterilised distilled water (1000 con/mL), were used for the experiment. The assay was performed in the presence or absence of Tramesan oligos in the concentration of 100 µM and 200 µM solutions dissolved in water, using 96-wells microplates. The cultures were incubated at 30˚C for 3 days. The assay allowed testing all the fractions in minimal amount and to generate hundreds of replications in a very short time (aflatoxin microtitre-based bioassay). Different cultures were independently filtered with Millipore filters (0.22 µm). The extraction of AFB_1 and OTA was performed with

chloroform/methanol (2:1, v/v), using 5 µL of Quercetin (purity ≥ 95% Sigma-Aldrich) 100 µM as recovery standard, as previously reported (Fanelli et al., 2000). The mixture was vortexed for 1 min, centrifuged and then the lower phase was collected. The extraction was repeated three times and the samples concentrated under a N_2 stream; AFB_1 was re-dissolved in 50 µL of acetonitrile/water/acetic acid (20:79:1 v/v) whereas OTA in 50 µL Methanol and quantified by triple quad LC/MS 6420 (Agilent). The amount of AFB_1 was evaluated by using an ISTD-normalised method in MassHunter workstation software, quantitative analysis version B.07.00. Aflatoxin B_1-13C-d3 (Clearsynth) at 2 µM final concentration was used as ISTD. AFB_1 and OTA amounts were expressed in ppb.

2.3 Results

2.3.1 Purification and characterization of oligosaccharide fractions

The polysaccharide fraction was purified according to the procedure described in the Methods and analyzed by size exclusion chromatography (SEC) to separate possible fractions at different molecular masses. The chromatogram pinpointed two fractions: fraction B1 and fraction C1 (Fig 1). Two further chromatograms from other polysaccharide amounts showed very similar shapes and the three B fractions were pooled together as well as the C fractions. These pooled fractions were thereafter named B and C.

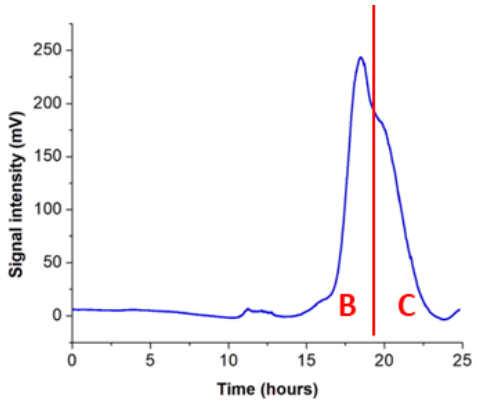


Figure 1. SEC analysis of the polysaccharide obtained from the culture filtrate of *T. versicolor*

Part of fractions B and C were dialysed, lyophilised, excanged with D_20 for the NMR analysis. The NMR spectra of the B and C fractions, prior to hydrolysis, showed that they are very similar if not identical in compostion (Fig.2.)

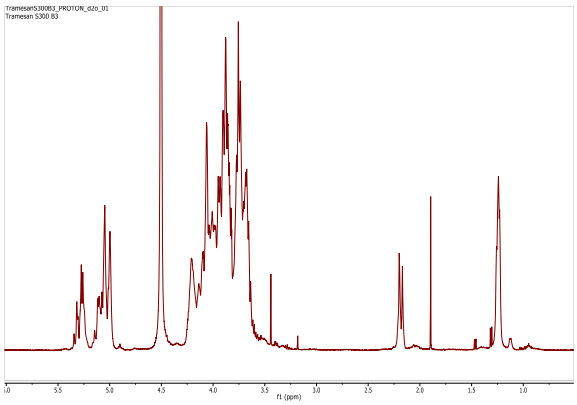


Figure 2. [1]H NMR spectra of the B fraction (a) and C fraction (b) obtained from *T. versicolor* culture filtrate.

In order to get Tramesan oligomers still representative of the polymer repeating unit and suitable for biological activity test, the fractions B and C were subjected to partial hydrolysis and the resulting products were separated by SEC on a Bio Gel P2 column in order to recover the obtained oligosaccharides. The chromatograms obtained from the hydrolisates B and C are shown in Fig. 3 a and b, respectively. The two chromatograms are almost identical, a part differences in sample concentration, indicating the similarity of the B and C native fractions.

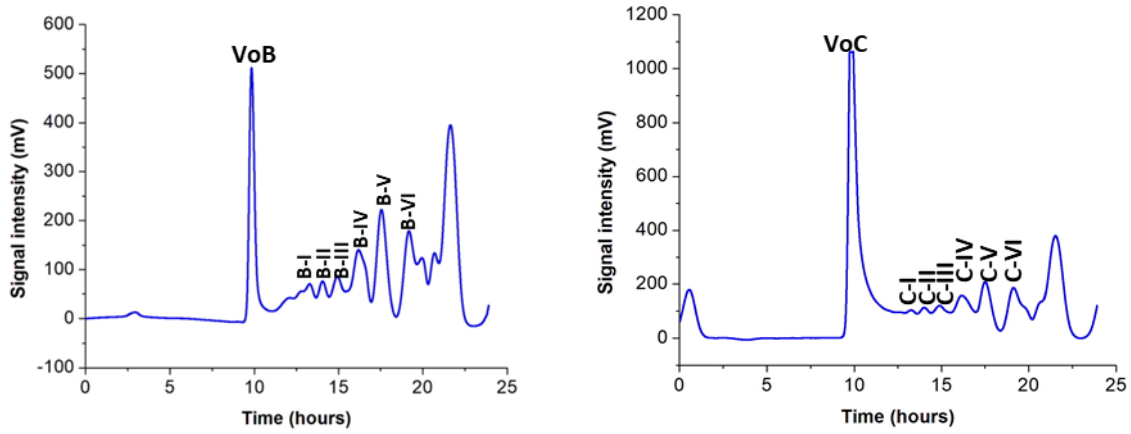


Figure 3. a) Chromatogram of the hydrolisate from the B fraction. b) Chromatogram of the hydrolisate from the C fraction

Disregarding the VoB and VoC fractions, which eluted at the void volume and relay to high mass oligomers and/or polysaccharide, fractions B and C showed six different peaks with decreasing molecular mass, and a more intense peak corresponding to the totally retained species, which include salts. The fractions corresponding to the six peaks indicated in Fig. 3 were called B-I to B-VI and C-I to C-VI, respectively, for the native B and C fractions, and they

were analysed by ESI-MS and NMR spectroscopy to determine the structure of the oligosaccharides contained in each of them.

ESI-MS gave information on the size of oligosaccharides and their composition (i.e. hexoses or deoxy-hexoses). Fragmentation of single ions (data not shown) indicated that Fuc is always terminal. NMR spectroscopy gave information on the type of sugars present and their relative molar ratio. Fraction B-IV was studied for a detailed NMR analysis using 1-D and 2-D spectra. It resulted to be composed of two tetrasaccharides: one containing only Gal and the second one composed of Man ridues with terminal reducing Fuc. All the investigated oligosaccharide fractions were similar to B-IV but different in size.

2.3.2 Definition of structure of oligosaccharides

(a) *ESI-MS analysis*

All the B oligosaccharide fractions were identical to the co-respective C fractions. Therefore, the following ESI-MS data analysis is focused only on B fractions. The oligosaccharides contain only Man, Fuc and Gal residues in perfect agreement with the results published in a previous publication (Scarpari et al., 2017).

Tramesan fraction B VI contained a mixture of disaccharides: Gal-Gal and Man-Fuc. Since MS only disguishes between Hex and deoxy-Hex residues, the definition of Gal and Man resideus was obtained from the previous publication (Scarpari et al., 2017) and confirmed by NMR proton spectra. A similar analysis carried out on the B V fractions showed that it is composed of two trimers, Gal-Gal-Gal and Man-Man-Fuc. The remaning B fractions (B IV to B I) were composed of two tetramers, pentamers, hexamers, and heptamers. As an example, the ESI-MS spectrum of fraction B II, which is composed of two hexamers, is reported in Fig. 4.

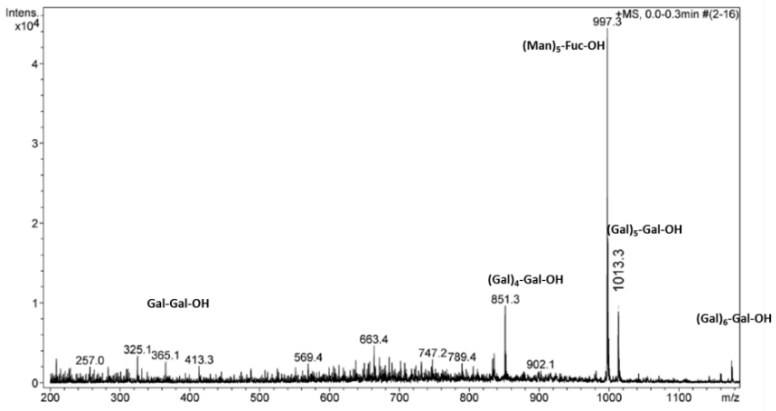


Figure 4. ESI-MS spectrum of fraction B II obtained after hydrolysis of the polysaccharide purified from the *T. Versicolor* culture filtrate.

(b) NMR analysis

The NMR analysis of the B and C fractions prior to hydrolysis showed that they are very similar, if not identical, in composition. Considering the MS results about composition of the fractions collected after hydrolysis (B I to B VI), the discussion of the NMR data is hereafter reported only for the B fractions after hydrolysis. In addition, more details on the NMR analysis will be given for fraction B IV taking into account that this fraction could be considered as representative of all the collected fractions after hydrolysis, differing only in molecular masses. Fraction B VI and B V contain two dimers and the NMR proton spectrum confirmed the MS data but permitted to define also the identity of the monosaccharides in the dimers (Gal-Gal and Man-Fuc for fction B VI, and Gal-Gal-Gal and Man-Man-Fuc for fraction B V) in good agreement with previous findings (Scarpari et al., 2017). As mentionad, fraction B IV contains a mixture of two tetramers and the spectrum is shown in Fig. 5

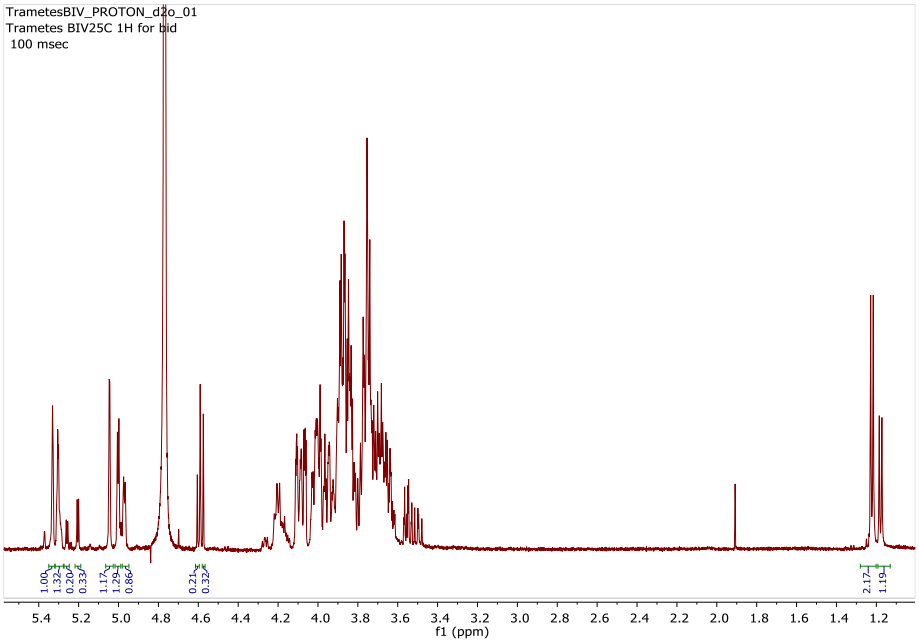


Figure 5. ^{1}H NMR spectrum of fraction B IV.

In the spectrum, the signals in the 5.4 – 4.4 ppm range are relative to anomeric protons, a part the intense signal at about 4.8 which refers to the residual partially deuterated water (DHO). The two doublets at 1.2 ppm are attributed to the methyl groups of the Fuc residue and the fact that the methyl group gives origin to two signals reflects the equilibrium between the two reducing forms of the sugar (α-Fuc and β-Fuc). The same multeplicity is also present for the anomeric protons of the reducing ends of the Gal containing tetramer and the $(Man)_3$-Fuc tetramer, as reported in Fig. 6 where the expansion of the anomeric proton's ppm range is

reported. In addition, the presence of two sugars at the reducing end confirmed that two oligosaccharides are present in the mixture.

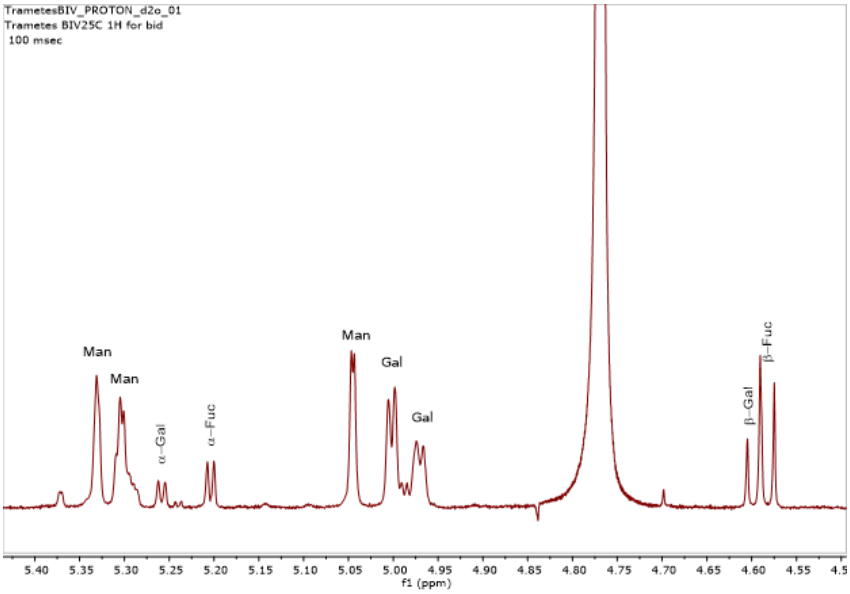


Figure 6. Anomeric proton's ppm range of the ^{1}H NMR spectrum of fraction B IV.

The occurrence of anomeric signals in the 4.95 – 5.35 ppm range indicate that, a part the anomeric equilibrium of the reducing sugar, all the non-reducing monosaccharides are in the α configuration; while the signal integration values indicate that the $(Man)_3$-Fuc oligomer is present in higher amount with respect to the $(Gal)_4$ one. The analysis of 2D NMR spectra permitted the identification of the monosaccharides present in the oligomers and the assignement of the carbons involved in the glycosidic bonds. This information was contained in the COSY and HSQC spectra, respectively, reported in Fig. 7 and 8.

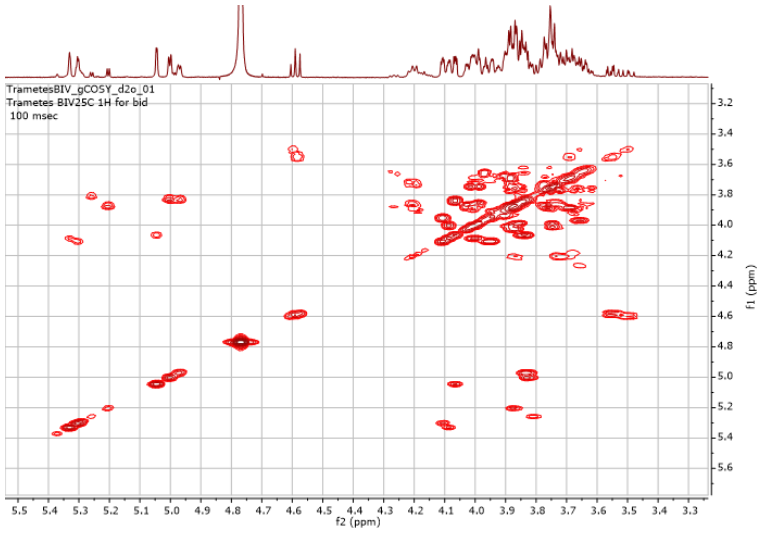


Figure 7. 2D NMR COSY spectrum of fraction B IV

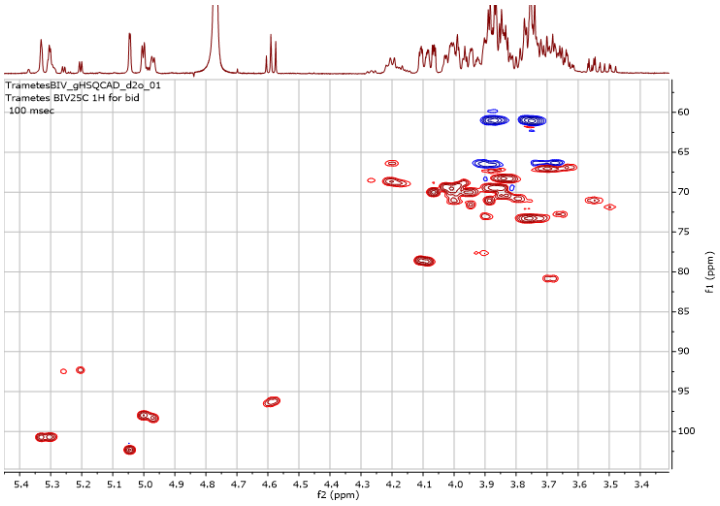


Figure 8: 2D NMR HSQC spectrum of fraction B IV

Specifically, in the COSY spectrum the three cross peaks at 3.33-4.08 ppm, 5.30-4.10 ppm, and 5.05-4.06 ppm, which refer to the H1 – H2 cross magnetization, are typical of Man residues, while the three cross peaks at 5.26-3.81 ppm, 5.00-3.83 ppm, and 4.97-3.82 ppm, again caused by the H1-H2 cross magnetization, are typical of Gal residues,thus allowing a precise identification of the monosaccharides constituting the oligomers. In the HSQC spectrum, which reports the cross-magnetization between carbon atoms and their bound protons, the shift at high ^{13}C ppm (vertical scale) of the signals relative to the H2 protons od Man residues (assigned in the COSY spectrum and occurring at 4.10-78.64 ppm and 4.08-78.64 ppm) indicate that Man residues are connected by a 1-2 glycosidic bond. Similarly, the shift at higher ppm values of the C6 carbon signals of the Gal residues (66.49 ppm and 66.25 ppm), with respect to the conventional 61.00 ppm, indicates the Gal residues are connected throughout a 1-6 glycosidic bond. A similar information was obtained by analysing the spectra of all the remaining B fractions obtained after hydrolysis and the chemical structure of the oligomers constituting each fraction, and obtained from MS and NMR data, is reported in Table 1

Table 1: Structure of the oligomers present in the Tramesan fractions after acidic hydrolysis of the native polymer

Tramesan fractions:	Composition:	Type of oligosaccharide*:
B VI and C VI	mixture of disaccharides	αMan-Fuc-OH and αGal-Gal-OH
B V and C V	mixture of trisaccharides	$(\alpha Man)_2$-Fuc-OH and $(\alpha Gal)_2$-Gal-OH

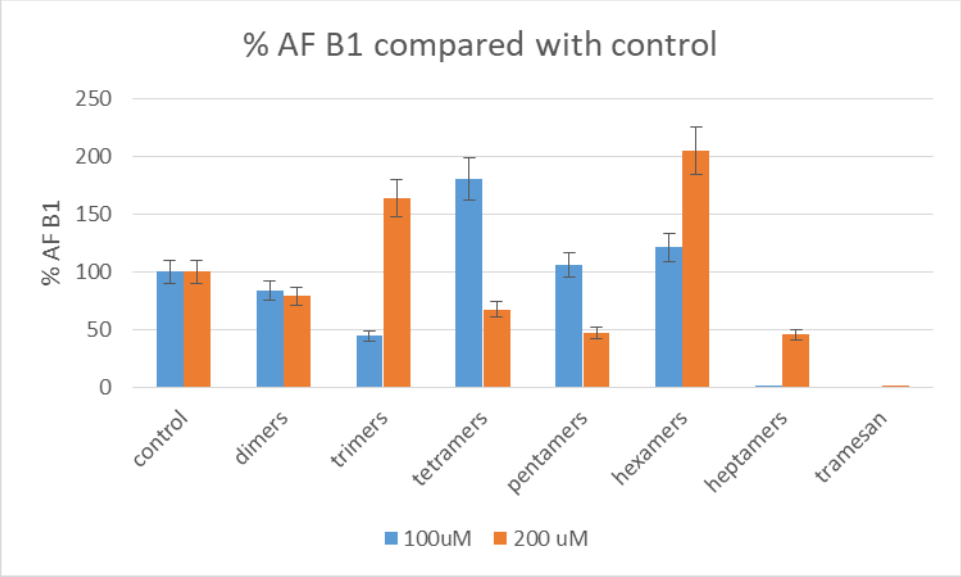
B IV and C IV	mixture of tetrasccharides	$(\alpha Man)_3$-Fuc-OH and $(\alpha Gal)_3$-Gal-OH
B III and C III	mixture of pentasaccharides	$(\alpha Man)_4$-Fuc-OH and $(\alpha Gal)_4$-Gal-OH
B II and C II	mixture of hexasaccharides	$(\alpha Man)_5$-Fuc-OH and $(\alpha Gal)_5$-Gal-OH
B I and C I	mixture of heptasaccharides	$(\alpha Man)_6$-Fuc-OH and $(\alpha Gal)_6$-Gal-OH

* All Man residues are 1-2 linked; all Gal residues are 1-6 linked.

2.3.3 Mycotoxin inhibition assay for oligosaccharides of Tramesan

Each oligosaccharide fraction was tested for inhibition of mycotoxin production by *A. flavus* 3357 and *A. carbonarius* using a 96-well microtitre plate assay. Since oligosaccharide amounts were very low the concentration of 100 µM and 200 µM solutions of the oligosaccharides dissolved in water were used. Double concentrated potato dextrose broth (48g/L), 100 µL of mixture (water solution of different oligosaccharides), and 10µL of conidial suspension (1000con/mL) were used for the experiment. The results, reported in Figure 9, showed that oligosaccharides longer than 7 units showed the highest rate of mycotoxin inhibition, while the smaller ones had no significant biological activity.

A



B

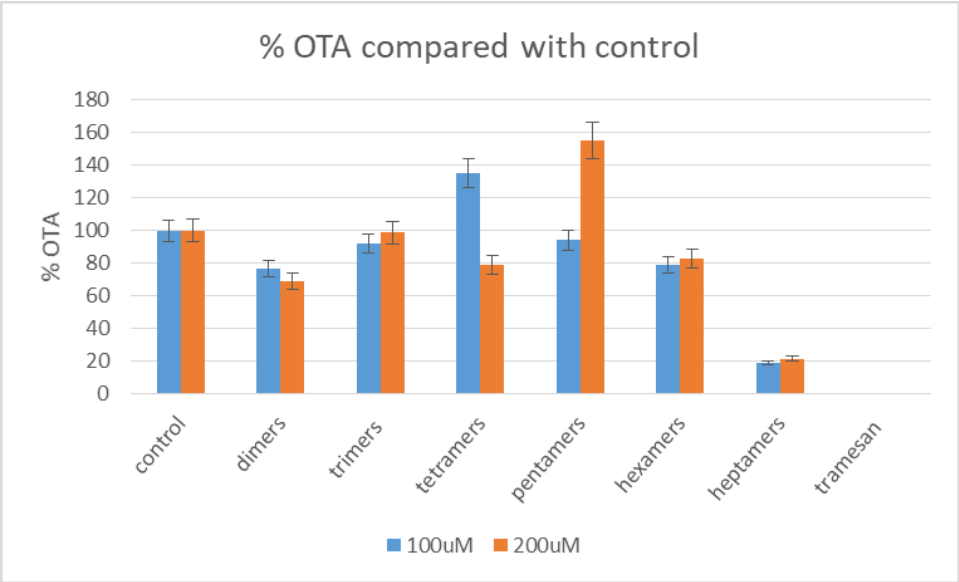


Figure 9 A and B. Relative inhibition by different oligosaccharides and Tramesan of Aflatoxin B_1 and Ochratoxin A production by *A.flavus* NRRL 3357 and *A. carbonarius* strain. Bars represent the mean ± SD of three determinations of five separate experiments.

2.4 Discussion

Polysaccharides derived from mushroom metabolites showed the potential to becoming a control agent for mycotoxin production (Zjalic et al.,2006; Scarpari et al., 2014; 2017, Reverberi et al., 2011). One of the most promising biocontrol producers is the edible non-toxic basidiomycete *T.versicolor*. Previous studies indicated the potential activity of extracts from culture filtrate of mycelia of *T. versicolor* and its characterised polysaccharide Tramesan©, in controlling the growth and secondary metabolism (e.g. mycotoxins) of plant pathogenic fungi. Furthermore, studies have shown that the bioactivity of Tramesan© relies mostly on its ability to act as a pro-antioxidant molecule, regardless of the biological system on which it was applied (Scarpari et al., 2017). The partial structure of Tramesan indicates that it is predominantly α-glucan, although we cannot exclude a part of chain that has not yet been characterized. Additionally, the polysaccharide of *T. versicolor* contains a total of 61,194 g/100g dm of all glucans, of which 0.25% refers to α-glucans and 37% refers to β- glucan (Sari et al., 2017). Therefore, we can assume that the active part of the polysaccharide is not yet fully characterized, and that it probably includes a beta glycoside bond. Although the positive properties of Tramesan have been proven, due to changing environmental conditions, random mutations may occur, which could alter the structure of the active polysaccharide moiety. The

purified exopolysaccharide of *T. versicolor* was partially hydrolyzed and separated by column chromatography into fractions from disaccharides to heptasaccharides.

Each fraction was individually tested (100uM and 200uM concentrations) for AFB_1 inhibition production by *A. flavus* and OTA control in *A. carbonarius*. Fragments smaller than 7 units showed no significant effect on mycotoxin inhibition, whereas heptasaccharides showed inhibitory activity up to 90% (100 μM) and 55% (200 μM) in *A. flavus* and up to 81% (100 μM) and 78% (200 μM) in *A. carbonarius*. Since all of the oligosaccharides are composed of a mixture of two oligosaccharides of the same length but with different composition, at present it is not possible to determine which one, of the two fractions, is active, or if both have to be present. Besides, synergistic or antagonistic effects of the two different heptamers from the mixture on aflatoxin synthesis cannot be excluded. In future research, it will be necessary to separate the heptamers in order to characterize the exact structure of the active moiety and to avoid the potential interaction of two different heptamers. Specifically, for di, three, penta and hexasaccharides, a higher concentration of oligosaccharides has shown to promote aflatoxin synthesis. Also, the production of OTA was enhanced by the presence of tetramers and pentamers. This could be explained by the use of oligosaccharides as carbohydrate supplementary source, which in turn favours mycotoxin synthesis (Fanelli and Fabbri, 1981). Furthermore, the reported results indicate that a lower concentration of heptasaccharide is more efficient in inhibiting the synthesis of aflatoxins. This could be due to differences in the structure (predominantly α-glucan), and to the fact that at higher concentrations the oligosaccharides could degrade into smaller fragments, some of which may stimulate aflatoxin synthesis. In particular, aflatoxin production is highly influenced by the identity and concentration of available carbon sources such as; certain types of sugars, fatty acids, sterols and synthetic triglicerides (Fabbri et al., 1980; Fanelli et.al, 1981, 1983; Passi et al., 1984; Castoria et al., 1989; Luchese et al., 1991; Nesci et al., 2011). For instance, Davis and Diener (1967) studied the aflatoxin production and growth of *Aspergillus parasiticus* using various carbon sources and concluded that, generally, compounds that are normally oxidized through both the hexose monophosphate and the glycolytic pathways, supported both growth and aflatoxin production. However, oligosaccharide concentration and aflatoxin inhibition are not always directly correlated, as evidenced in the results obtained using tetrasaccharide fractions, reported in Table 2. This phenomenon was already reported in previous studies on mycotoxin inhibition (Fanelli and Fabbri, 1980). In fact, these may be dose dependent actions, in which we have an increased effect of low concentration of sugar on aflatoxin synthesis. In addition,

heptasaccharides, that is the largest fragments of the polysaccharide investigated, have shown the greatest influence on the control of mycotoxin synthesis. From these results, we can assume that the active polysaccharide fraction is greater than seven units, and that a lower concentration of heptaccharides has a better effect on aflatoxin and ochratoxin inhibition (on account of the aforementioned possible degradation of oligosaccharides, which then stimulates mycotoxin production). These results are also consistent with previous research on the correlation between polysaccharide size and their activity in mycotoxin inhibition. Moreover, Scarpari et al. (2016) proved that semi-purified culture filtrate of *T. versicolor* containing molecules with a supposed molecular mass >3.0 kDa, showed a special ability in enhancing antioxidant activity in *A. flavus*, by drastically inhibiting the biosynthesis of aflatoxins. Thus, we might assume that the size of the oligosaccharides is important for the binding to a specific receptor in the cell wall, and that longer oligosaccharides are more likely to bind and elicit a cell response. Since it is proven that larger parts of oligosaccharides are active in mycotoxin control, the characterization of heptamers is ongoing and the study of the active structure is underway. In addition, in the future it will be necessary to produce octa and nanosaccharides and to test their biological activity in controlling the mycotoxin production. This research indicates some novel insights on the active structures of Tramesan oligosaccharides, which could result in the synthetic production of the active moiety and then in its widespread use in agriculture. In conclusion, the active part of the *T. versicolor* polysaccharide could be a possible new eco-friendly tool for mycotoxin control.

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Chapter 3. Effect of mushroom culture filtrates on inhibition of mycotoxins produced by *Aspergillus flavus* and *Aspergillus carbonarius*

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Abstract

Mycotoxins are secondary metabolites produced by certain fungi that have hazardous effects on humans and animals, which in turn result in disease and economic losses. Two of the mycotoxins of greatest public health and agroeconomic significance are aflatoxin B_1 (AFB_1), and ochratoxin A (OTA). There is a need for the development of natural compounds for the control of these mycotoxins in food and feed. There has been interest in the use of mushroom polysaccharides for controlling mycotoxins. In this study 42 isolates of different ligninolytic mushrooms were assayed for their ability to inhibit the synthesis of OTA and AFB_1. The results showed that 23 strains produce metabolites able to inhibit the synthesis of OTA by >20%, and 32 strains that produced metabolites that inhibited AFB_1. Only two strains, *Trametes versicolor* strain TV117 and *Schizophyllum commune* strain S.C. Ailanto, produced metabolites able to significantly inhibit (>90%) the synthesis of both mycotoxins. It is known that *T. versicolor* produces a polysaccharide Tramesan, able to inhibit the synthesis of AFB_1 and was probably responsible for mycotoxin inhibition. The results suggest that *S. commune* metabolites were able to inhibit both OTA and AFB_1 synthesis may be based on polysaccharides.

3.1 Introduction

Mycotoxins are low-molecular-weight compounds synthesized by filamentous fungi, which are toxic to vertebrates (Ciegler and Benneth, 1980). The first mycotoxins, aflatoxins, were discovered in 1960 (Blount, 1961), since then about 400 molecules were classified as mycotoxins . The effect of mycotoxins on humans and animals vary according to their chemical structure and can go from cytotoxic to carcinogenic effects (Benneth and Klich, 2003; Kniewald, 2013; Santos Pereira et al., 2019). Aflatoxins, ochratoxins, trichothecenes, zearelenone, fumonisins, and ergot alkaloids are the mycotoxins of the greatest agro-economic importance. Some toxigenic fungi have the ability to produce more than one mycotoxin (e.g. *Fusarium* spp.) and some mycotoxins are produced by species from more than one fungal genus (e.g., *Aspergillus* and *Penicillium* species). Often, more than one mycotoxin is found on a contaminated food commodities (Hussein and Brasel, 2001). Due to the severity of the toxic effect and persistence during the transformation approx. a dozen mycotoxins have strict legislative limits for food and feed use. Among these aflatoxins (AFs) and ochratoxin A (OTA) are important in a range of economically important commodities. Both mycotoxins are synthetized by species of the genus *Aspergillus.* The species of this genus are xerophilic and able to grow under water stress conditions, much wider than those allowing plant growth. They can thus colonise and contaminate a range of food commodities both pre- and post-harvest (Khatoon and Abidin, 2018). Both AFs and OTA are very stable and heat resistant during food/feed processing. Furthermore, both can be stored in some animal tissues and transmitted along the food chain (Bryden, 2007). Moreover, AFs are classified as carcinogenic (group 1A) by the IARC, International Agency for Research on Cancer (IARC, 2012) while ochratoxin A (OTA) is classified as a possible carcinogen for humans, group 2B (IARC, 1993). The presence of these mycotoxins is regulated by EC regulation 1881/2006. Different strategies to control the presence of mycotoxins in food and feed have been applied for many years, but none of them have been completely successful. Thus, there is a need for minimisation and prevention control strategies for these mycotoxins. Many strategies have been predominantly based on the use of chemical compounds (pesticides and fungicides). However, the EU has removed almost 50% of the chemical groups used in crop protection and there is thus a need for more environmentally beneficial natural control compounds.

The requirement of products with lower impact on the environment and on human health, able to control mycotoxin production, has increased. The research on natural compounds able to

control mycotoxin presence in food and feed is constantly growing in the last decades (Yin et al., 2008; Bianchini and Bullerman, 2009; Gómez et al., 2018). Among the assayed natural compounds mushroom metabolites have been shown to be a potentially more environmentally friendly tool for mycotoxin control. Metabolites of several mushroom species are reported to be able to control mycotoxin synthesis to some extent. These metabolites were mostly identify as polysaccharides. The most studied ones in mycotoxin control are those of *Lentinula edodes* and *Trametes versicolor*, two ligninolytic mushrooms, known also for the production of biologicaly active polysaccharides used in medical trials (Wasser, 2002, 2004; Akgul et al., 2017). Exo-polysaccharides of *L. edodes* showed high inhibiting effects towards OTA and AFs, while Tramesan, a polysaccharide produced by *T. versicolor*, showed a high (almost total) and long lasting inhibition of AF biosynthesis (Reverberi et al., 2005, 2011; Scarpari et al., 2015, 2017,). Most of the research until now conducted with mushroom metabolites were oriented towards the inhbiting effects on single mycotoxins. In this study a wide number of ligninolytic mushrooms isolates present in the collection of the Laboratory of Plant Pathology and Mycology of Sapienza University of Rome were screened for their capacity to produce metabolites able to control simultaneously the biosynthesis of both AFs and OTA.

3.2 Materials and methods:

3.2.1 Fungal strains and mushrooms used in this study

A.flavus (Speare) (NRRL 3357), producer of aflatoxin B_1, was cultured on PDA (Potato Dextrose Agar), at 30°C for 7 days in dark conditions, and a suspension of 1×10^6 conidia per mL in sterilized distilled water was used as an inoculum.

A. carbonarius, a producer of ochratoxin A, was isolated by the Laboratory of Plant Pathology and Mycology of Sapienza University of Rome. This was grown on PDA at 30°C for 7 days in dark conditions, and a suspension of 1×10^6 conidia per mL in sterilized distilled water was used as an inoculum.

Table 1. shows the list of mushroom species and strains from which extracts were made for screening and efficacy against *A.flavus* and *A.carbonarius*.

Table 1. Different mushroom isolates used for the experiment.

Code	Genus	Species
CF 16	*Agrocybe*	*aegerita*
CF 249	*Agrocybe*	*aegerita*
CF 250	*Agrocybe*	*aegerita*
CF 278	*Armillaria*	*mellea*
CF 253	*Fomes*	*fomentarius*
CF 255	*Fomes*	*fomentarius*
CF 262	*Fomes*	*fomentarius*
P.COLLEGNO 2A	*Ganoderma*	*adspersum*
COLLETTA 12	*Ganoderma*	*adspersum*
ROBUR (69.2)	*Ganoderma*	*adspersum*
Meisino 9	*Ganoderma*	*adspersum*
Tiglio	*Ganoderma*	*adspersum*
Cep.10	*Ganoderma*	*adspersum*
Diano 2	*Ganoderma*	*adspersum*
CF 17	*Ganoderma*	*lucidum*
CF 223	*Ganoderma*	*lucidum*
CF 264	*Ganoderma*	*lucidum*
DP1	*Ganoderma*	*resinaceum*
PLATANO ROMA	*Ganoderma*	*resinaceum*
P.VERDI 920	*Ganoderma*	*resinaceum*
780.2	*Ganoderma*	*resinaceum*
DP63	*Ganoderma*	*resinaceum*
ASPROMONTE 52	*Ganoderma*	*resinaceum*
DP23	*Ganoderma*	*resinaceum*
C.TRENTO 34 B	*Ganoderma*	*resinaceum*
P. VERDI 4A	*Ganoderma*	*resinaceum*
G.R. 853	*Ganoderma*	*resinaceum*
CF 258	*Ganoderma*	*resinaceum*
CF 6	*Grifola*	*frondosa*
CF 19	*Grifola*	*frondosa*
CF 225	*Heterobasidion*	*annosum*
CF 224	*Phellinus*	*pfefferi*
CF 25	*Pleurotus*	*eryngii*
CF 38	*Pleurotus*	*eryngii*
CF 28	*Polyporus*	*sulphureus*
BELLAGIO 2	*Schizophyllum*	*commune*
DP61	*Schizophyllum*	*commune*
GTM8 R1	*Schizophyllum*	*commune*
S.C. AILANTO	*Schizophyllum*	*commune*
CF 18	*Stropharia*	*rugosoannulata*
CF 280	*Trametes*	*hirsuta*
T.V. 117	*Trametes*	*versicolor*

3.2.2 Preparation of mushroom exo-polysaccharides

The 42 isolates of different mushroom cultures detailed above were kept on Potatoe dextrose agar (PDA, Himedia, India) medium at 4°C and the cultures were sub-cultured onto fresh medium every 30 days. Three plugs of 1 cm diameter of each isolate were inoculated in 100 mL of Potato Dextrose Broth (PDB, Himedia,India), and incubated for 15 days at 25°C under shaken conditions (100 rpm). Then, the liquid cultures were homogenized, in sterile conditions. After homogenization, an aliquot (5% v/v) of the fungal cultures, were inoculated in 500 mL of PDB in 1L-Erlenmeyer flasks and incubated for 21 days at 25°C under rotary shaken conditions (100 rpm). After incubation, the mycelia were separated from the culture filtrates by subsequent filtrations through 0.45 μm filters (Sartorius, Goettingen, Germany), to eliminate all the mycelia. Mycelia-purified culture filtrates were lyophilized and utilized for subsequent analyses as reported in Zjalić et al. (2006). Finally, concentrations (2% w/v) of each mushroom polysaccharide unrefined substrates were utilized for the experiment.

3.2.3 *In vitro* screening of mushroom polysaccharides on inhibition of mycotoxin synthesis by *Aspergillus flavus* and *Aspergillus carbonarius*

For the purpose of wide screening of mushroom polysaccharides the microtiter-based bioassay using 96-wells microplates, was performed. This assay allowed the testing of all the fractions using small quantities of the compounds with effective replication (n-10) over short periods of time.

Screening assays were carried out with 100 conidia of each strain (*A. flavus* and *A. carbonarius*), independently, in 10 μL of sterilized distilled water. This was inoculated together with 190 μL of PDB, in presence or absence of Tramesan and each of the other lyophilized polysaccharides in each of the wells. The microtiter plates were incubated at 30°C for 3 days in dark conditions. Different cultures were independently filtered with Millipore filters (0.22 μm, Sartorius, Goettingen, Germany). The filtrate was subsequnetly used for mycotoxin extraction.

3.2.4 Mycotoxin quantification

(a) Extraction of Ochratoxin A

The different cultures were independently filtered through Millipore filters (0.22 μm, Whatman). The extraction of OTA was performed, for each condition (control and treated with 2% of different polysaccharides), using the extraction solution of CH_3CN: H_2O: CH_3COOH (79:20:1 v/v), with the addition of 5 μL of Quercetin (≥ 95% Sigma-Aldrich) 100 μM as an internal standard, as previously reported in Fanelli et al. (2000). The mixture was vortexed for 1 min, centrifuged and then the lower phases was drawn off. The extraction was repeated three times and the samples were concentrated under a N_2 stream and re-dissolved in 50 μL methanol. Lastly, the concentration of OTA was determined by HPLC/MS (Agilent, Waldbronn, Germany). The concentration of OTA was expressed in ppb.

(b) Extraction of Aflatoxin B_1

The AFB_1 was extracted by adding Chloroform: Methanol (2:1 v/v) with the addition of 5 μL of Quercetin (≥ 95% Sigma-Aldrich) 100 μM as an internal standard (Fanelli et al., 2000). The mixture was vortexed for 1 min, centrifuged and then the lower phases were drawn off. The extraction was repeated three times and the samples were concentrated under a N_2 stream, then re-dissolved in 50 μL methanol. AFB_1 was quantified by HPLC/MS (Agilent, Waldbronn, Germany). The amount of AFB_1 was expressed in ppb.

3.2.5 Mycotoxin inhibition assay with *Schizophyllum commune* unrefined extract

The extract of *S. commune* was tested for inhibition of AFB_1 and OTA by the *A. flavus* and *A. carbonarius* strains using the same assay format detailed previously. Concentrations of 0, 5% w/v, 1% w/v, and 2% w/v of *S. commune* extract were used, dissolved in Potatoe Dextrose Broth (PDB), and compared with the control (PDB). In both experiments the procedure described previously was used. The plates were incubated at 25°C for three days in dark conditions, as described in Parroni et al. (2019).

3.2.6 Comparison between commercial pure Schizophyllan and extract of *S. commune* on inhibition of mycotoxin synthesis

The inhibition of mycotoxin production in *A. flavus* and *A. carbonarius* was compared between the unrefined extract of *S. commune* and commercial β-glucan Schizophyllan (Invivogen, France), using the microtiter plate assay described previously. The mycotoxin analyses was as described previously. The statistical analysis was performed using the Student t test, $P < 0.05$.

3.3 Results

3.3.1 Screening of mushroom polysaccharides on inhibition of aflatoxins and ochratoxin A synthesis.

The growth of the toxigenic species was not significally affected by the presence of almost all lyophilised filtrates of different mushrooms, except for *Schizophyllum commune* (GTM8 R1), *Ganoderma asdpersum* (Tiglio), and *Pleurotus eryngii* (CF 25) (data not shown). Although these strains partially inhibited mycelium growth, toxin production was not significantly inhibited.

The percentage (%) control of OTA and AFB_1 biosynthesis in the presence of 2% of unrefined lyophilized filtrates of the different ligninolytic mushrooms are shown in Table 2. For the purpose of this research the mean inhibiting rates <20% were considered non- significant. Most of the mushroom extracts showed inhibitory effects on AFB_1 (32 out of 42), with less effective in controlling OTA (23 out of 42). The great variability in inhibitory effects could be observed amongst different isolates of the same species. Figure 1 shows the effect of different isolates of *Ganoderma adspersum*. All strains had some inhibitory effects on AFB_1, while the effect on OTA was less effective. Also, different strains of *G. lucidum* showed different inhibitory potential in controlling AFB_1 and OTA biosynthesis. One strain, CF17, had little influence on the synthesis of both mycotoxins, while the the second CF223 showed some effects on both (19.8% OTA, 20% AFB_1) while the third, CF264, inhibited AFB_1 production by >50% and OTA by approx. 30% (Figure 3). The biggest differences were observed among the isolates of *Schizophyllum commune.* Four isolates were used in the experiments. Strain DP61 had no significant effect on mycotoxin control. The second strain, GTM8 R1, showed a slight inhibition of AFB_1 (27%), but no inhibition of OTA. The third strain, BELLAGIO 2, inhibited

the synthesis of both mycotoxins by about 50%. The fourth one, S.C. Ailanto, gave the highest inhibition, with >90% control of both mycotoxins (see Figure 3). In Figure 4 (a, b), 5 isolates that gave the best inhibitory effects against both mycotoxins are shown. It is evident that the only two strains able to produce metabolites that were able to inhibit the synthesis of both mycotoxins were *T. versicolor* TV 117 and *S. commune* (S.C Ailanto). All the other assayed strains produce compounds able to inhibit the synthesis of one of the mycotoxins but having little or no influence on the synthesis of the other.

Among the mushroom species, the unrefined extract of the *S. commune* (S.C. Ailanto) showed the highest inhibitory effect for the control of both mycotoxins (AFB_1 and OTA), with the exception of the strain *T. versicolor* 117.

Table 2. Inhibition rate (%) of different fungal isolates on aflatoxin B_1 and ochratoxin A production. Inhibitions <20% were not considered significant for the purpose of this research and are reported as not significant (NS). All data are the mean ± SD of five determinations of five separate experiments ±SE.

Code	Genera	Species	Inhibition of OTA(%)	STDEV	Inhibition of AF (%)	STDEV
CF 16	*Agrocybe*	*aegerita*	NS		69.5	± 7.6
CF 249	*Agrocybe*	*aegerita*	31.3	± 3.8	62	± 7.4
CF 250	*Agrocybe*	*aegerita*	23.5	± 3.3	58.6	± 8.2
CF 278	*Armillaria*	*mellea*	NS		26	± 2.6
CF 253	*Fomes*	*fomentarius*	40	± 6	20	± 3
CF 255	*Fomes*	*fomentarius*	33	± 4.6	57.6	± 8
CF 262	*Fomes*	*fomentarius*	NS		43.7	±5.2
P.COLLEGNO 2A	*Ganoderma*	*adspersum*	25.7	± 3.3	NS	
COLETTA 12	*Ganoderma*	*adspersum*				
ROBUR (69.2)	*Ganoderma*	*adspersum*	NS		69.8	± 7.6
Meisino 9	*Ganoderma*	*adspersum*	NS		54.3	± 5.4
Tiglio	*Ganoderma*	*adspersum*	26	± 3.12	28.8	± 3.5

Cep 10	*Ganoderma*	*adspersum*	NS		65.8	± 9.2
Diano 2	*Ganoderma*	*adspersum*	26.7	± 3.5	56	± 7.3
CF 17	*Ganoderma*	*lucidum*	NS		NS	
CF 223	*Ganoderma*	*lucidum*	NS		20.2	± 2.2
CF 264	*Ganoderma*	*lucidum*	30	± 3	54	± 5.4
DP 1	*Ganoderma*	*resinaceum*	20	± 2.6	23	± 2.9
PLATANO ROMA	*Ganoderma*	*resinaceum*	31.8	± 3.8	NS	
P.VERDI 920	*Ganoderma*	*resinaceum*	41.8	± 5.8	25	± 3.5
780.2	*Ganoderma*	*resinaceum*	44.7	± 5.8	26	± 2.8
DP 63	*Ganoderma*	*resinaceum*	42.8	± 5.1	35.8	± 5.3
ASPROMONTE 52	*Ganoderma*	*resinaceum*	57.9	± 8.1	31.6	± 3.2
DP 23	*Ganoderma*	*resinaceum*	NS		NS	
C.TRENTO 43 B	*Ganoderma*	*resinaceum*	30	± 4.2	NS	
P.VERDI 4A	*Ganoderma*	*resinaceum*	NS		NS	
G.R. 853	*Ganoderma*	*resinaceum*	NS		28	± 3
CF 258	*Ganoderma*	*resinaceum*	NS		42.5	± 6.4
CF 6	*Grifola*	*frondosa*	NS		NS	
CF 19	*Grifola*	*frondosa*	NS		24	± 3.1
CF 225	*Heterobasidion*	*annosum*	NS		21.8	± 3
CF 224	*Phellinus*	*pfefferi*	NS		NS	
CF 25	*Pleurotus*	*eryngii*	26.8	± 3.2	NS	
CF 38	*Pleurotus*	*eryngii*	70	± 9.8	22	± 3.2
CF 28	*Polyporus*	*sulphureus*	25	± 2.5	NS	
BELAGIO 2	*Schizophyllum*	*commune*	43	± 5.2	47.9	± 6.7
DP61	*Schizophyllum*	*commune*	NS		NS	
GTM8 R1	*Schizophyllum*	*commune*	NS		27	± 4

S.C. AILANTO	*Schizophyllum*	*commune*	94.8	± 6.6	95.3	± 8.2
CF 18	*Stropharia*	*eugosoannulata*	NS		38	± 4.9
CF 280	*Trametes*	*hirsuta*	NS		20.8	± 2.1
T.V. 117	*Trametes*	*versicolor*	98.2	± 11.8	98.8	± 9

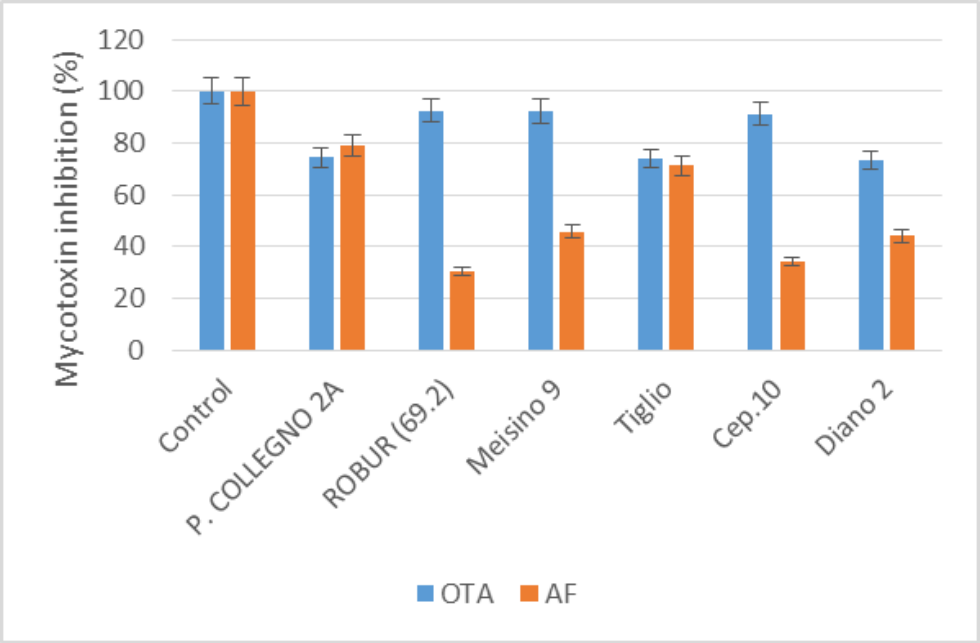


Figure 1. The effect of different isolates of mushroom *Ganoderma adspersum* lyophilized rough filtrates on ochratoxin A production in *A. carbonarius* and aflatoxin B_1 production in *A. flavus* after 3 days incubation at 30°C. Results are expressed as percentage of mycotoxins produced relative to the control. The data are the mean ± SD of three determinations of five separate experiments.

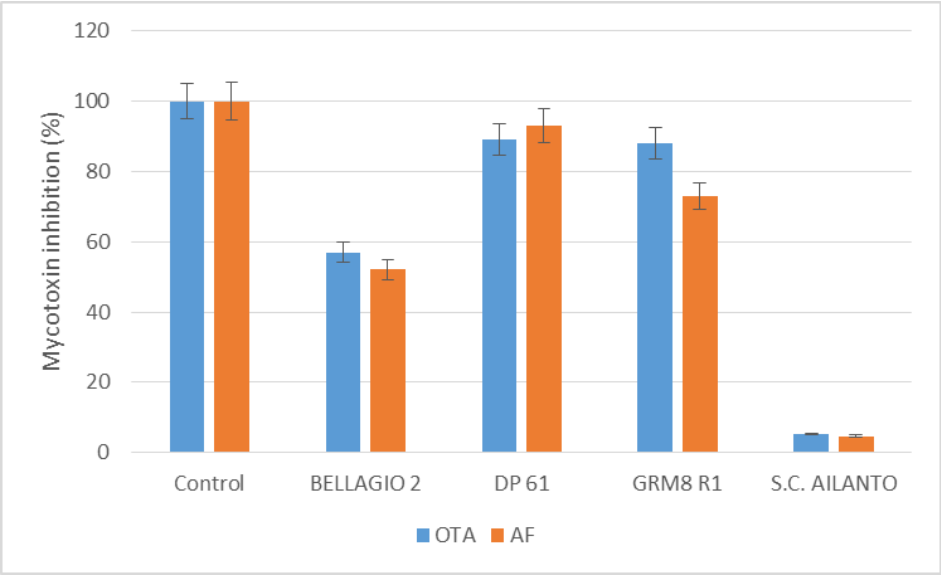


Figure 2. The effect of different isolates of the mushroom *S. commune* lyophilized filtrates on ochratoxin A production by *A. carbonarius* and aflatoxin B_1 production by *A. flavus* after 3 days incubation at 30°C. Results are expressed as percentage of mycotoxin relative to the control. The data are the mean ± SD of three determinations of five separate experiments.

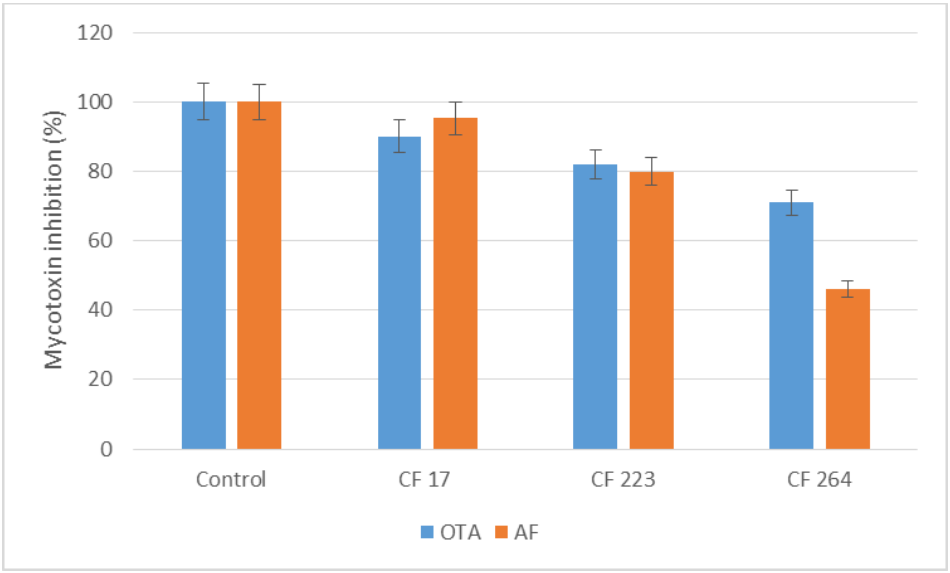
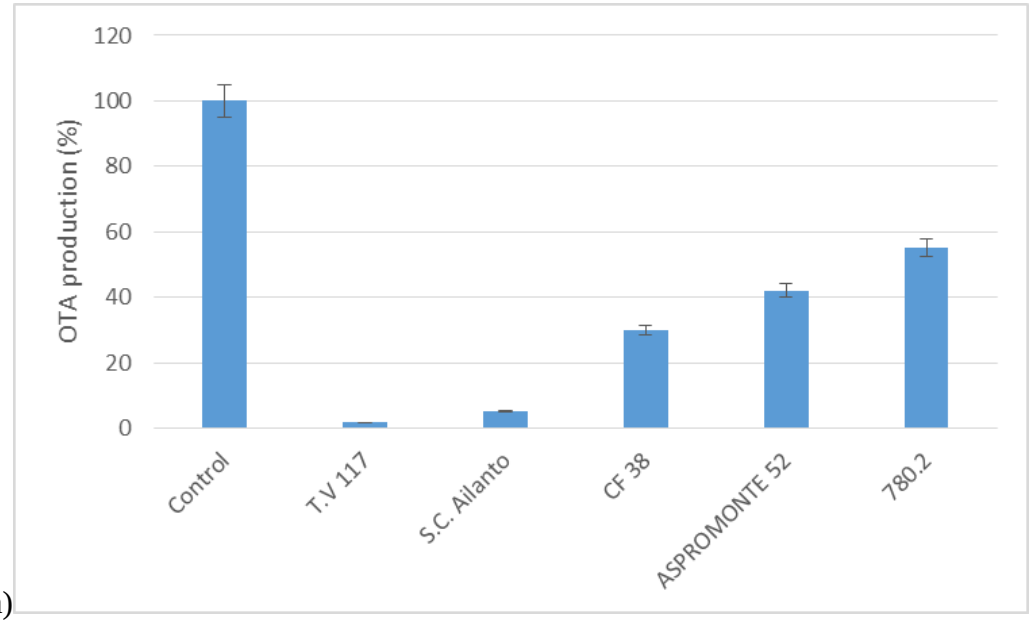


Figure 3. The effect of different isolates of the mushroom *Ganoderma lucidum* lyophilized filtrates on ochratoxin A production in *A. carbonarius* and aflatoxin B_1 production in *A. flavus* after 3 days incubation at 30°C. Results are expressed as percentage of mycotoxins produced relative to the control. The data are the mean ± SD of three different determinations of five separate experiments.

a)



b)

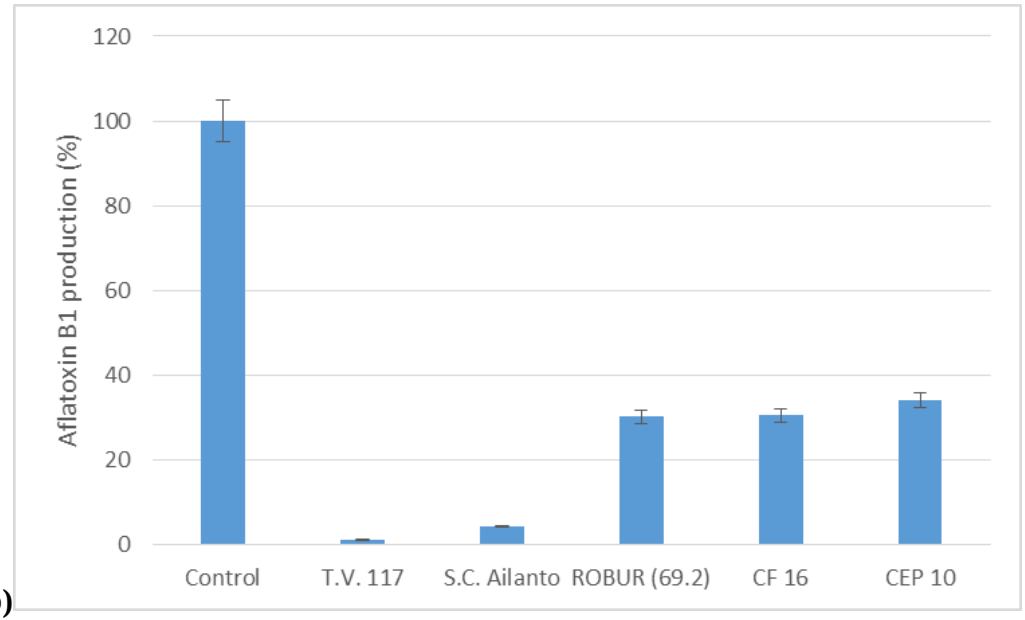


Figure 4. The extracts of the 5 isolates of different species of mushrooms that gave the best inhibitory effects for control of both aflatoxin B_1 and ochratoxin A**.** Results are expressed as percentage of mycotoxins produced relative to the control. The data are the mean ± SD of three different determinations of five separate experiments.

The effects of the polysaccharides of *T. versicolor* on inhibition of AFB_1 and OTA has previously been reported. Therefore the research focused on *S. commune* isolate S.C. Ailanto. Figure 5. shows the results where different concentrations of the unrefined extract of this mushroom were used. The concentration of 0.5% of unrefined extract inhibited the synthesis of AFB_1 by >80% and that of OTA by >75%. The higher concentration of 1%, inhibited the synthesis of both mycotoxins by almost 95% while the highest concentration assayed, 2%, inhibited production by 98% for both toxins.

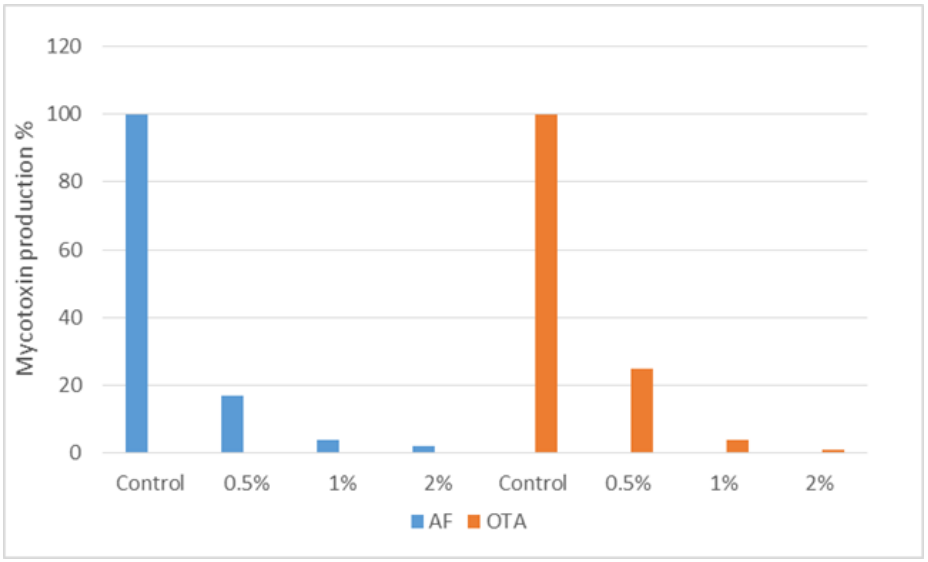


Figure 5. The effects of different concentrations (0.5, 1 and 2% w/v) of unrefined extract of *S. commune* isolate S.C. Alianto on the biosynthesis of aflatoxin B_1 by *A. flavus* and ochratoxin A by *A. carbonarius.* Results are expressed as percentage of mycotoxins produced relative to the control. The data are the mean ± SD of five determinations of three separate experiments.

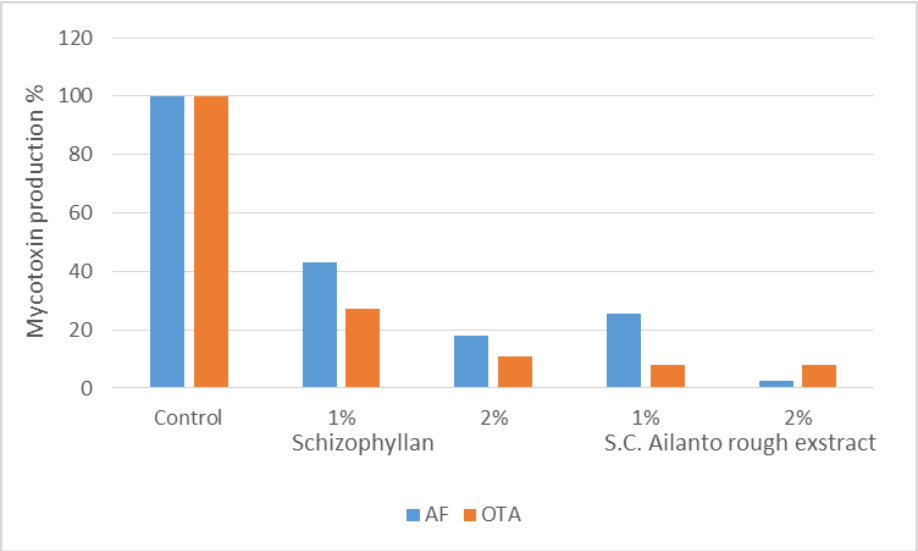


Figure 6. Comparison of the inhibition of biosynthesis of aflatoxin B1 and ochratoxin A in treatments of different concentrations of the commercial Schizophyllan polysaccharide and *S. commune* isolate S.C. Ailanto unrefined extract after 3 days incubation. Results are expressed as percentage of mycotoxins produced relative to the control. The data are the mean ± SD of five determinations of three separate experiments.

S. commune is a known producer of a bioactive polysaccharide schizophyllan. This polysaccharide is characterized and commercially produced. To verify the possibility of the involvement of this polysaccharide in the inhibition of the biosynthesis of AFB_1 and OTA. Figure 6 shows the effect of the addition of two concentrations (1 and 2% w/v) of the Schizophyllan and the unrefined extract of *S. commune* isolate S.C. Ailanto (LF) on the synthesis of the two mycotoxins under study. Both treatments showed inhibitory potential of both mycotoxins. The inhibition obtained with the use of LF was high at both concentrations and for both mycotoxins when compared to those obtained with the commercial Schizophyllan. In fact, at the concentration of 1% unrefined extract inhibited the synthesis of AFB_1 by 75% and OTA by 90%. The commercial compound had inhibitory effects of 60% and 70% respectively. Similar results were obtained with the 2% unrefined extract when compared to the commercial product.

3.4 Discussion

The results of this research showed that several basidiomycete fungal species produced metabolites which were able to control the synthesis of these two mycotoxins to some extent. The variability in inhibition rates among the different isolates of the same species is consistent with the results obtained previously by Reverberi et al. (2005) and Zjalic et al. (2006). These variations could depend on genetic structure of the strain and its capacity to produce inhibiting compound(s), or could be a consequence of different environmental adaptations of the strains, and active compounds produced under different environmental conditions (temperature, pH, levels of oxygenation). The most studied inhibiting effects of mushroom metabolites are those made with *Lentinula edodes* (Ricelli et al., 2002; Reverberi et al., 2005, 2011) and *Trametes versicolor* (Zjalic et al., 2006, Reverberi et al., 2010, Scarpari et al., 2014; 2017,) using lyophilized culture filtrates. In both cases the inhibiting compound was a polysaccharide, and, when applied to *A. parasiticus*, it acted through an increase in anti-oxidant production in the cells of the mycotoxigenic species. In *A. parasiticus*, this enhancement involved the Yap protein (Reverberi et al., 2008) whereas in *A. ochraceous* its homologue Yaop (Reverberi et al., 2012). Given the similar mechanism of action of mushroom polysaccharides studies so far, the present study screened a number of lyophilized culture filtrates for efficacy and control of mycotoxins.

The results obtained suggest that other molecules than polysaccharides produced by the assayed mushrooms could be involved in inhibitory effects of one of the two studied mycotoxins. In fact, it is known that different anti-oxidants can interfere with the synthesis of both AFB_1 and OTA (Barberis et al., 2009). The inhibition of one of the two mycotoxins, or marked differences in inhibition between them, could be a result of the presence of some anti-oxidant compounds (e.g., phenolic compounds) which may be more active in the inhibition of one of the two mycotoxins. The only lyophilized culture filtrates that gave significant control of both mycotoxins (>90%), were those of *T. versicolor* strain TV117 and *S. commune* strain S.C Ailanto. It was previously known that strain TV117 produced a polysaccharide named Tramesan and that it was active in aflatoxin inhibition. Thus, in the present study, the work was focused on the metabolite of *S. commune*. It was postulated that the active compound was probably a polysaccharide. This was subsequently confirmed by the experiments with schizophyllan, a polysaccharide produced by *S. commune*, which is also commercially available. In fact, this polysaccharide showed potential to control the synthesis of both mycotoxins after 3 days incubation, when liquid fermentation had probably reached the

stationary phase. Schyzophillan is a large polysaccharide of several hundreds of kDa, and its activity depends on its configuration (Kidd, 2000). Perhaps, other molecules produced by the mushroom are necessary to obtain the right configuration. However, further studies are needed on the possible involvement of schizophyllan in the inhibition of AFB_1 and OTA synthesis to clarify whether this polysaccharide is predominantly responsible for toxin inhibition. In addition, the role and nature of other *S. commune* metabolites which may be involved, should be examined. Amongst all isolated mushroom polysaccharides, the unrefined extract isolated from *S. commune* (S. C. Ailanto), revealed that the extracts of this strain were the best in inhibiting both mycotoxins. The comparison between the inhibition of mycotoxin biosynthesis by means of commercial β-glucan Schizophyllan and *S. Commune* (S.C. Ailanto) crude extract, showed a higher inhibition rate for the unpurified S. C. Ailanto extract. This effect could probably be due to different compounds present in the unpurified extract, (such as proteins), with different inhibitory impacts. Furthermore, the strain S. C. Ailanto extract exhibited the highest inhibition rate of both mycotoxins at a concentration of 2% w/v. This suggests that some larger oligosaccharides or perhaps polysaccharides are active in blocking mycotoxin synthesis, which requires further research. Previous studies suggests that β-glucans are proven to be pharmacologically active compounds that could stimulate antioxidant responses in animal and fungal cells (Mizuno, 1995; Islam et al., 2019; Kidd, 2000). Moreover, fungal β -glucans could be amongst the factors responsible for the inhibiting effect of aflatoxins (Reverberi et al., 2005), perhaps acting as free radical scavengers (Slamenova et al. 2003). Schizophyllan (SPG), is a neutral extracellular polysaccharide first discovered by Kikumoto et al., (1970). Its structure is well known and defined previously. Notably, SPG is a non-ionic, water-soluble homoglucan, which possesses a β-(1-3)-linked backbone with single β-(1-6)-linked glucose side chains at approximately every third residue, with a molecular weight of 450 kDa (Zhang et al., 2013). SPG is secreted in submerged culture medium by the wood-rotting and filamentous growing basidiomycete *Schizophyllum commune*, and has numerous potential applications, such as: thickener for cosmetic lotions, oxygen-impermeable films for food preservation, high-value pharmaceutical applications and can also act as a biological response modifier (BRM) and non-specific stimulator of the immune system (Rau, 2004; Zhang et al, 2013). It has been reported that most of the antitumor polysaccharides such as SPG, show the same basic β -glucan structure with different types of glycosidic linkages. In particular, the β -glucans containing mainly (1-6)-linkages have less antitumor properties than the ones containing β-(1-6)-branch points (Franz, 1989). Moreover, high molecular weight glucans appear to be more effective than those of low molecular weight (Jong et al., 1991; Sakagami et al.,1991; Zhang et al., 2013). Kojima

et al. (1986) on their part, reported that high molecular weight (4 100, 000) SPG forms a triple helix with a helical symmetry, and that only the triple helical schizophyllan is active. Conversely, lower molecular weight (50, 000) SPG has neither the triple helix structure nor any antitumor activity. These studies are consistent with our results, which confirms that a higher concentration of S. C. Ailanto had a higher efficiency on mycotoxin inhibition in *A. flavus* and *A. carbonarius*. In addition, it could be suggested that, besides showing the antioxidant activity per se, the S.C. Ailanto lyophilized raw filtrates influence the cascade of signals which allows mycotoxin biosynthesis, even if we can not indicate at which step of the pathway this event occurs. Nevertheless, it could be hypothesised that β-glucans and other compounds contained in raw filtrate of S.C. Ailanto, could be able to inhibit mycotoxin synthesis in *A. flavus* and *A. carbonarius* by enhancing the internal antioxidant system. Nevertheless, future research is required to determine the specific part of the polysaccharide active in the inhibition of AFB_1 and OTA, so that it can be synthetically produced and used for large scale applications in agriculture. In particular, the discovery of the very active part of the polisaccharide would be especially relevant, from an economic point of view, as its synthesis would be more cost-effective in comparison with commercial Schizophyllan. Moreover, fungal extracts could represent a promising tool in mycotoxin control compared to chemicals or natural products as plant extracts (Mahoney and Molyneaux, 2004; Sanchez et al., 2005), due to their low toxicity when released into the environment.

In conclusion, the *S. commune* isolate studied in this research could be regarded as a potential agent in biological control or as a useful component among the integrated strategies against mycotoxin producing fungi in food and feed products. Besides, since the assayed mushroom contains nutritive and active compounds, the addition of schizophyllan β-Glucan in food and feed could contribute to enhance their nutritional value. Lastly, β-Glucans are able to beneficially affect different target functions in animals in a such way that the state of well-being, health and resistance to disease are improved (Fanelli et al., 2004; Zjalić et al., 2006).

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Chapter 4. Preliminary results of the influence of *Schizophyllum commune* polysaccharides on the inhibition of mycotoxin synthesis in *Aspergillus flavus* and *Aspergillus carbonarius*

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Abstract

There has been interest in the use of mushroom polysaccharides to control the synthesis of key mycotoxins in staple commodities. Tramesan(c), an exo-polysaccharide produced by *Trametes versicolor*, is among the most efficient and studied ones. The objective of the present study were to analyse the effects of secreted polysaccharides of *Schizophyllum commune* on production of aflatoxin B_1 (AFB_1) and ochratoxin A (OTA) produced by *Aspergillus flavus* and *Aspergillus carbonarius* respectively. Comparison of the effects of commercial schizophyllan and partialy purified *S. commune* polysaccharides indicated that the inhibition of AFB_1 and OTA synthesis may be due to other types of polysaccharide(s) rather than schizophyllan. Preliminary results suggest that the mechanism of efficacy of the *S. commune* polysaccharides could be analogous to that found previously for Tramesan®, i.e., by enhancing the antioxidant response in the target fungal cells.

4.1 Introduction

Mycotoxins are low molecular weight compounds produced as a secondary metabolites by a wide range of different fungi, especially *Aspergillus, Penicillium* and *Fusarium* (Rodrigues and Naehrer, 2012). They are commonly found in the terrestrial ecosystem and thus able to contaminate crops, both pre-harvest and post-harvest (Varga and Toth, 2005). Some of the factors that influence the colonisation of staple food crops and mycotoxin contamination are the prevailing weather conditions, relative humidity, moisture content, especially during ripening and post-harvest drying, temperature, insect damage, the timing and use of fungicides and how good agricultural practices are implemented**.** Some studies suggests that under environmental or chemical stress these fungal species fungi produce mycotoxins under stress conditions such as nutritional imbalance and drought stress (Reverberi et al, 2010; Rodrigues and Naehrer., 2012; Hong et al., 2013;Scarpari et al., 2015;). Climate change interacting abiotic factors may also impact on the production and contamination of staple foods by mycotoxins. (Medina et al., 2017). Although the relationship between climate change and fungal growth is not yet fully established, it is known that changes in temperature, humidity, and precipitation can cause „stress“ conditions and stimulate mycotoxin production by mycotoxigenic fungi (Medina et al., 2017). While there are geographic and climatic differences in the occurrence and production of mycotoxins, exposure to this substances is a worldwide problem, and crops are most commonly contaminated with more than one mycotoxin (Magan and Olsen, 2004). Moreover, aflatoxins are predominantly associated with commodities of sub-tropical and tropical origin, but their occurrence has also been reported also in temperate climates (Pettersson et al, 1989). Aflatoxins predominantly contaminate oilseeds, edible nuts and cereals (Coker, 1997). Due to their hazardous effect on human and animal health the International Agency for Research on Cancer have classified aflatoxin as carcinogenic to humans (Group 1) (IARC, 1993). For this reason, aflatoxins are strictly limited by European laws (EC/1881/2006) (Scarpari et. al., 2016). Furthermore, Ochratoxin A represents a severe health hazard for humans and animals (WHO, 2011). OTA contamination occurs in a variety of food and feed such as coffee beans, spices, meat , cheese product and wine (El Khoury and Atoui, 2010). Moreover, based on a large body of evidence of carcinogenicity detected in several animal studies the IARC evaluated the carcinogenic potential of OTA as a possible human carcinogen (Group 2B), (WHO-IARC, 1993) (Kupier-Goodman, 1999). Many countries have adopted regulations to reduce the possible dietary intake, thereby preserving the health of animals and consumers (for Europe: EC No 1881/2006, EU No 165/2010, EU No 105/2010). However, the allowed levels

of contamination are not harmonized among countries, and this may cause trade frictions at the global level (Dellafiora et al., 2017). Due to their high resilience and resistance to high temperatures, mycotoxins cannot be completely removed from food and feed, and therefore strategies to prevent mycotoxin synthesis in toxigenic fungi are required. Use of chemicals, mainly with fungotoxic and fungostatic properties, were the most common methods used in agriculture for prevention of mycotoxin contamination. The awareness that different chemicals, could present both environmental problems, health hazard and the development of resistant strains, has led to large limitation of their use. Since 2014 European Community (EC) has banned about 50% of chemicals used in agriculture.

Previous research has shown that exo-polysaccharides from different mushrooms, (mainly Basidiomycota), have shown some potential for the control of fungal growth and mycotoxin production (Zjalic et al., 2006; Scarpari et al., 2015, 2017; Reverberi et al., 2011). One of the most promising basidiomycetes which producers such compounds is *Trametes versicolor*, an edible and species sometimes considered a "healling mushroom" for its efficacy against some pathologies (Ricciardi et al., 2017). The exo-polysaccharide has been purified, partially characterized and named Tramesan®. Tramesan is able to act as an inhibitor of mycotoxin synthesis in *Aspergillus flavus* (Scarpari et al., 2015; 2017). A range of basidiomycete species and strains were screened for the production of similar polysaccharides was carried out in Chapter 3. Their effect on the inhibition of mycotoxin synthesis in *A. flavus* and *A. carbonarius* was examined. Among all the fungi tested, the crude extract of exo-polysaccharide isolated from the mushroom *Schizophyllum commune* (S. C. Ailanto), showed the highest degree of control of biosynthesis of both mycotoxins. The exo-polysaccharide from *S. commune* has been studied previously and has been identified as schizophyllan (Zhang et al., 2013). Schizophyllan is a non-ionic, water-soluble homoglucan, which possesses an a β-(1-3)-linked backbone with a single β-(1-6)-linked glucose side chain at approximately every third residue (Kojima et al., 1986; Komatsu et al., 1969; Zhang et al., 2013). This polysaccharide possesses the ability to act as a biological response modifier (BRM) and non-specific stimulator of the immune system (Rau, 2002). Moreover, intracellular oxidants/antioxidant imbalances can be considered as a 'prerequisite' for mycotoxin production (Reverberi et al., 2010). Under the influence of „oxidative stress", the cell produces reactive oxigen species (ROS), such as superoxide anions, hydroxyl radicals, hydrogen peroxide, singlet oxigen and oxylipins which arise as by-products during metabolic processes, such as glucose respiration, and fatty acid metabolism. However, their poduction could also be caused by the influence of environmental stress (Halliwell and Gutteridge 2007a; Reverberi et al., 2010). Fungal cells can tolerate a certain amount of ROS by

increasing the synthesis of antioxidant molecules (e.g., α-tocopherols, ascorbic acid, carotene, reduced glutathione), and active enzymes [e.g., catalase (CAT), glutathione peroxidase (GPX) and superoxide dismutase (SOD)], to counteract the increased ROS concentrations (Halliwell and Gutteridge2007b; Reverberi et al., 2010).

The objectives of this study were to examine the efficacy of three different variants of polysaccharide derived from the mushroom *S. commune*, including commercial schizophyllan, semi-purified exo-polysaccharide, as well as the unrefined extract of the *S. commune* polysaccharide for inhibition of mycotoxin production by *A. flavus* and *A. carbonarius*. The potential effects of these compounds on antioxidant response in the fungal cells of these two species was examined.

4.2 Materials and methods

4.2.1 Fungal strains

A. flavus (Speare) (NRRL 3357), a producer of aflatoxin B1 (AFB_1) was cultured on Potato Dextrose Agar (PDA, HiMedia, India), at 30° C for 7 days in dark conditions, and a suspension of 10^6 conidia in 0.2 ml of sterilized distilled water was used as an inoculum in 25 ml of Potato Dextrose Broth (PDB, HiMedia, India), in the presence or absence of the *S. commune* lyophilized unrefined extract.

A. carbonarius was obtained from the collection of fungal strains of the Laboratory of Plant Pathology, Department of Environmental Biology, University Sapienza, Rome. The producer of ochratoxin A (OTA), was cultured on PDA at 30°C for 7 days in dark conditions, and a suspension of 10^6 conidia in 0.2 ml of sterilized distilled water was used as inoculum in 25 ml of PDB in the presence or absence of the *S. commune* unrefined lyophilized extract.

S. commune (S.C.Ailanto) was obtained from the collection of fungal strains of the Laboratory of Plant Pathology, Department of Environmental Biology, University Sapienza, Rome. The strain was sub-cultured on potato dextrose agar (PDA) in 9 cm Petri dishes, incubated at 25°C in dark conditions for 7 days. These plates were used to obtain 1 cm diameter culture plugs which were used to inoculate 250-ml Erlenmeyer flasks containing 100ml of (PDB) and incubated at 25°C on a rotary shaker (180rpm) for 7 days. The mycelia were then separated from the culture filtrates by filtration through different size filters (Whatmann) to eliminate all

the mycelia. Mycelia-purified culture filtrate was concentrated in rota-vapor (Rotavapor® R-300, Buchi, Essen city, Germany), and up the concentration of 100 mL. The process was repeated until 500 mL of concentrated culture filtrate was obtained as described in Parroni et al., (2019). Part of the culture filtrate was lyopholised and used for assays of *S. commune* crude filtrate on AFB_1 and OTA production. The rest of the culture filtrate was used for further purification and analysis of *S.commune* polysaccharides.

4.2.2 Purification of *S. commune* exo-polysaccharides

Concentrated culture filtrate was precipitated with Ethanol (99%), on ice, to remove the lipids. After the centrifugation (45 min/ 13000 rpm at 4°C), the pellet was resuspended with 1mL of 10 mM Potassium Phosphate Buffer (pH=7,5). After resuspension Proteinase K was added to the sample and left over night in shaking water bath on 37° C. During the night the sample turned to gel form. The sample was centrifuged (40 min/4000rpm, HERMLE, Z326K), but most of the sample remained in gel form, while only a small part of the fraction remained in the supernatant. Therefore Sample 1 (gel form) was separated from Sample 2 (supernatant) and separately subjected to dialysis (10 kDa) against the water. After 2 days of dialysis, the samples were lyophilized and utilized for subsequent analysis.

Sample 1 (375 mg) was rehydrated in 5 mL of H_20, the sample has formed a gel. To disrupt the triple helix of the schizophyllan structure the gel was heated for 4 hours at 140°C as desribed by Zhang et al. (2013). After the heating treatment, the gel took the form of a viscous solution.

The possible presence of proteins in both samples was checked by UV analysis. Sample 1 and Sample2 were dissolved separately in 2.4 mL of H_20 (the sample 2 was completely soluble in water) and both samples were diluted 100 times before analysis.

4.2.3 NMR spectroscopy

Sample 2 was exchanged two times with 99.9% D_2O by lyophilisation and then dissolved in 0.6 mL of 99.96% D_2O. Spectra were recorded on a 500 MHz VARIAN spectrometer operating at 25°C and 50°C. Standard parameters were used for 2D NMR experiments. Chemical shifts are expressed in ppm using the residual HOD signal as internal reference (4.76 ppm at 25°C and 4.5 ppm at 50° C). NMR spectra were processed using MestreNova software.

4.2.4 Assays of the different variants of *S. commune* polysaccharide on mycotoxin inhibition

(a) Assay of the crude substrate of exo-polysaccharide *S. commune* for control of aflatoxin B1 and ochratoxin A biosynthesis

The crude extract of exo-polysaccharide *S. commune* at a concentration of 1% (w/v) and 2% (w/v) was added to PDB (0.5 mL) and used as a nutrient medium for the cultivation of *A. flavus* and *A. carbonarius*. The nutrient medium with or without exo-polysaccarides (control), was inoculated with a conidial suspension of *A. flavus* in concentration 10^6 conidia in 0.2 ml of sterilized distilled water, or the suspension of 10^6 conidia in 0.2 ml of sterilized distilled water for *A. carbonarius*. PDB inoculated with *A. flavus* and *A. carbonarius* was used as a control. The samples were incubated for up to 6 days at 30°C in dark conditions.

(b) Assay of the semi-purified lyophilised polysaccharide fraction from *S. commune* (SAMPLE 1) on aflatoxin B1 and ochratoxin A biosynthesis

Semi purified exo-polysaccharide extracted from *S. commune* (Sample 1) at a concentration of 1% (w/v) and 2% (w/v) was added to PDB (0.5 mL) and used as a nutrient medium for the cultivation of *A. Flavus* and *A. carbonarius*. The experiment was divided into 2 parts. The nutrient medium was inoculated with a conidia suspension of *A. flavus* 10^6 conidia in 0.2 ml of sterilized distilled water, while in the second assay the suspension of 10^6 conidia in 0.2 ml of sterilized distilled water of *A. carbonarius* was used for inoculation. PDB inoculated with *A. flavus* or *A. carbonarius* was used as a control. The samples were incubated for 6 days at 30° C in dark conditions.

(c) Assay of the fraction of the polysaccharide from *S. commune* (SAMPLE 2) on ochratoxin A and aflatoxin B1 biosynthesis

The fraction (Sample 2) extracted from polysaccharide *S. commune* at a concentration of 1% (w/v) and 2% (w/v) was added to PDB (0.5 mL) and used as a nutrient medium for the cultivation of *A. flavus* and *A. carbonarius.* The nutrient medium with dissolved fraction of Sample 2 was inoculated with a conidia suspensions of the two test fungi using the same procedure as for Sample 1.

(d) Efficacy of the comemercial product Schizophyllan on ochratoxin A and aflatoxin B1 biosynthesis

Commercial Schizophyllan (Invivogen, France) at a concentration of 1% (w/v) and 2% (w/v) was added to PDB (0.5 mL) and used as a nutrient medium for the cultivation of *A. Flavus* and *A. carbonarius*. The experiment was divided into 2 parts. The nutrient medium was inoculated with a conidia suspension of *A. flavus* 10^6 conidia in 0.2 ml of sterilized distilled water, while in the second assay the suspension of 10^6 conidia in 0.2 ml of sterilized distilled water of *A. carbonarius* was used for inoculation. PDB inoculated with *A. flavus* and *A. carbonarius* was used as a control. The samples were incubated for 6 days at 30° C in dark conditions.

(e) Kinetics assay of *S. commune* crude filtrate on aflatoxin B1 and ochratoxin A biosynthesis over 24-72h.

The lyophylized crude extract of the polysaccharide from *S. commune* (2% w/v) was added to 25 ml PDB in 50-ml Erlenmeyer flasks subsequently inoculated with a conidia suspension of either *A. flavus or A. carbonarius.*. The flasks were incubated at 30°C for 12, 24, 48, 60, and 72 h in dark conditions. Fungal growth and AFB_1 or OTA production were quantified by weighing the mycelium after filtration (Millipore filters; 0.45-mm pore size). After weighing, the mycelium was stored at -60°C for the following analyses.

4.2.5 Mycotoxin qunatificiation

(a) Extraction of Aflatoxin B1 from culture media

For aflatoxin B1 extraction 2 ml of liquid culture were taken in sterile conditions from each sample, 5 µL of Quercetin (≥ 95% Sigma-Aldrich) 100 µM as Internal standard were added and than extracted (v/v) with Chloroform: Methanol (2:1 v/v) as previously described in Parroni (2019). The mixture was vortexed for 1 min, centrifuged and then the lower phases was drawn off. The extraction was repeated three times and the samples were concentrated under a N_2 stream, then re-dissolved in 50 µL Methanol. Aflatoxins B1 were monitored in the culture filtrates by HPLC/MS (Agilent, Waldbronn, Germany). The amount of aflatoxin A was expressed in ppb.

(b) Extraction of Ochratoxin A from culture media

For ochratoxin A extraction 2 ml of liquid culture were taken in sterile conditions from each sample, 5 µL of Quercetin (≥ 95% Sigma-Aldrich) 100 µM as Internal standard were added and than extracted (v/v) with solution CH3CN: H2O: CH3COOH (79:20:1 v/v) as described in Altiokka (2009). The mixture was vortexed for 1 min, centrifuged and then the lower phases was drawn off. The extraction was repeated three times and the samples were concentrated under a N_2 stream and re-dissolved in 50 µL Methanol. Lastly, the concentration of OTA was determined by HPLC/MS (Agilent, Waldbronn, Germany). The concentation of OTA was expressed in ppb.

4.2.6 Analysis of anti-oxidant enzyme activities in *A. flavus* and *A. carbonarius* treated and non treated with lyophilised raw filtrates of *S. commune*.

Analyses of the activities of the different anti-oxidant enzymes were performed according to the method of Reverberi et al. (2005) .

Superoxide dismutase (SOD) activity was evaluated by means of a spectrophotometric assay. In this competitive inhibition assay, superoxide generated by xanthine–xanthine oxidase was detected by monitoring the reduction of Nitrobleu Tetrazolium at 505 nm. Total SOD activity was measured at pH 7.8 in Tris– HCl 0.2 M and pH 10.0 in 50 mM Sodium Carbonate Buffer. One Unit of activity was defined as the amount of enzyme that yields 50% of maximal inhibition of Nitroblue Tetrazolium reduction by Superoxide.

Catalase (CAT) activity was measured by monitoring the decomposition of H_2O_2 that was followed by decrease in absorbance at 240 nm. The difference in absorbance at 240 nm per U per time was used as a measure for the catalase activity.

Glutathione peroxidase (GPX) was assayed by monitoring at 340 nm the decrease in absorbance due to the oxidation of NADPH at 37°C. The final concentration in the reaction mixture was 50 mM Potassium Phosphate Buffer, pH 7.0, 0.5 mM EDTA, 1 mM Reduced Glutathione,0.5 mM NADPH, 0.5 mM Sodium Azide, 0.24 U ml 1 Glutathione reductase and 150µM H_2O_2 . One unit of the enzyme oxidises 1µmol of NADPH min^{-1}.

4.3. Results

4.3.1 UV spectrum analysis of Sample 1 and Sample 2

The results obtained by analysing Sample 1 and Sample 2 under the UV spectrum proved no significant presence of proteins in the Sample 1 fraction (Fig. 1)

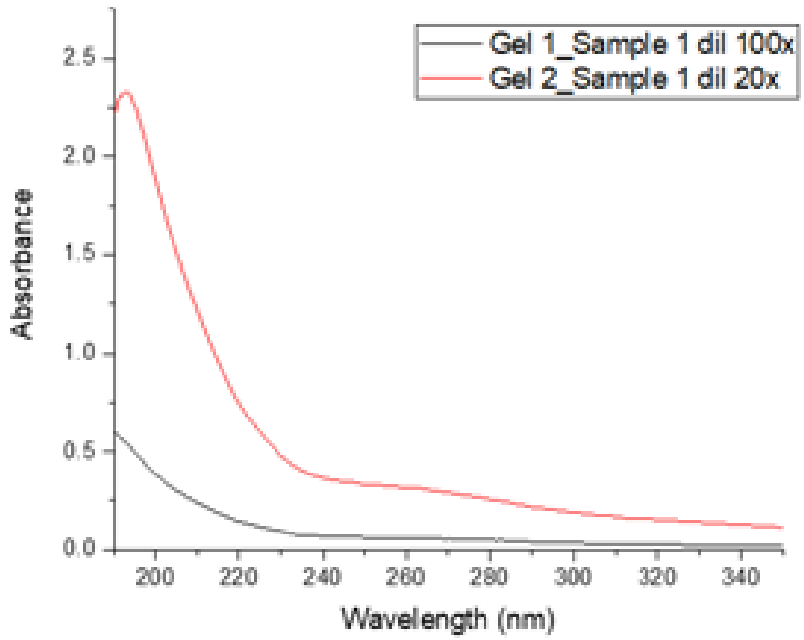


Figure 1. UV spectrum of Sample 1 and Sample 2 fractions

Sample 2 showed a much higher protein concentration than Sample 1 and was subjected to further protein precipitation using Proteinase K (Sigma- Aldrich).

4.3.2 1H NMR spectroscopy of Sample 2

The 1H NMR spectrum of Sample 2 was recorded at 25° C and 50° C and the spectra are shown in Figure 2. The structure of the repeating unit of scleroglucan is reported in Figure 3.

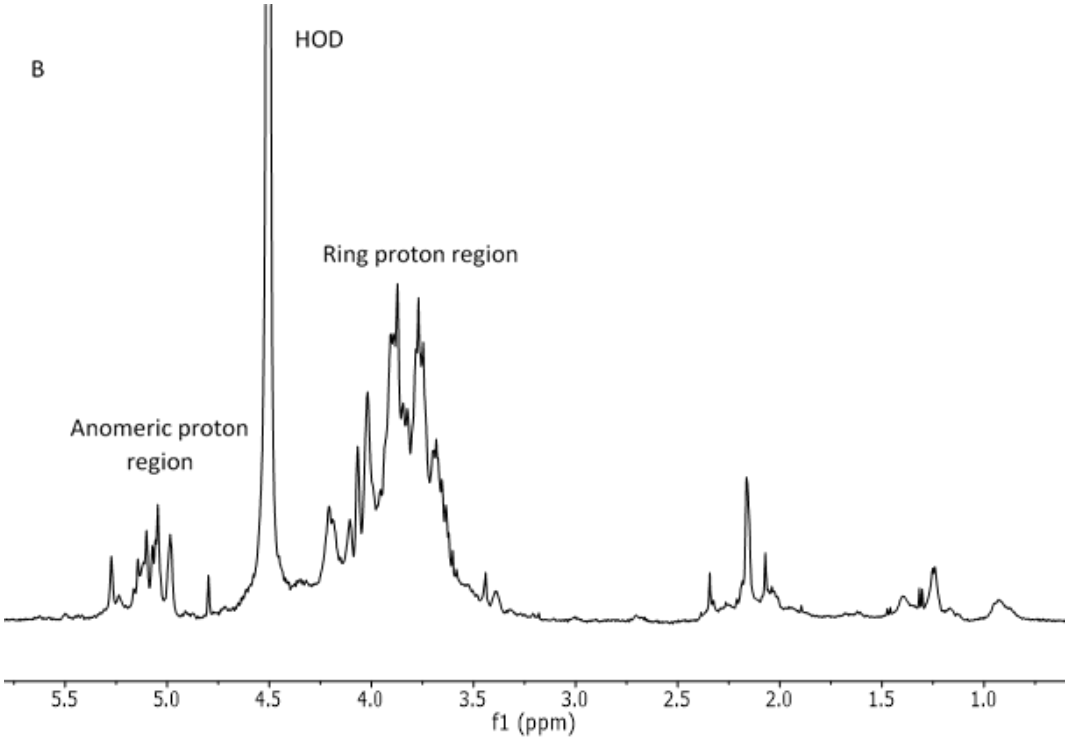


Figure 2. [1]H NMR spectra of Sample 2 recorded at 25° C (A) and 50° C (B).

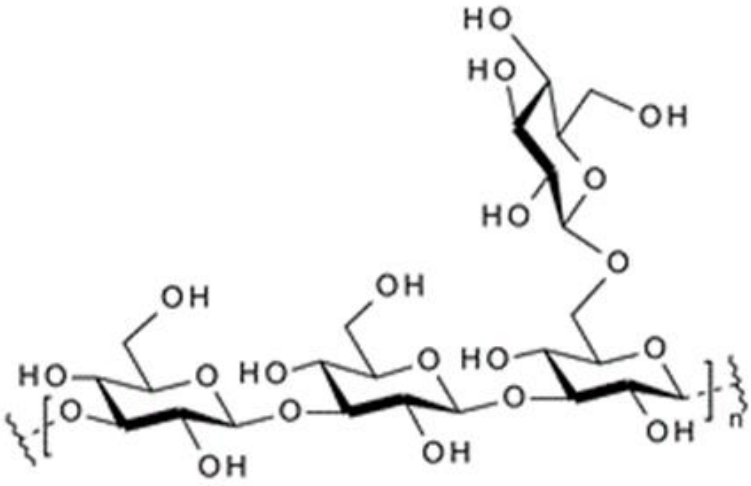


Figure 3. Structure of the repeating units of schizophyllan

Schizophyllan is composed of β(1-3) linked D-glucose with single unit side chains of β(1-6) glucose every third unit in the backbone. Since glucose is in the β-anomeric configuration, its H-1 protons are expected to have a chemical shift in the region 4.4 – 4.9 ppm. In fact, using the computer program CASPER to calculate ^{1}H NMR chemical shifts of schizophyllan repeating unit in D_2O, 4.5, 4.74 and 4.75 ppm values were calculated for its anomeric protons (Jansson et al., 2006). Although in the ^{1}H NMR spectrum of Sample 2 recorded at 50° C there is a very small signal at about 4.75 ppm, the expected signal at 4.5 is absent in the ^{1}H NMR spectrum recorded at 25° C, thus strongly suggesting that the polymer in Sample 2 has a saccharidic nature but that it is not schizophyllan.

4.3.3 Comparison of the commercial Schizophyllan and partially purified *Schizophyllum commune* exo-polysaccharide (Sample 1) on inhibition of aflatoxin B1 and ochratoxin A synthesis

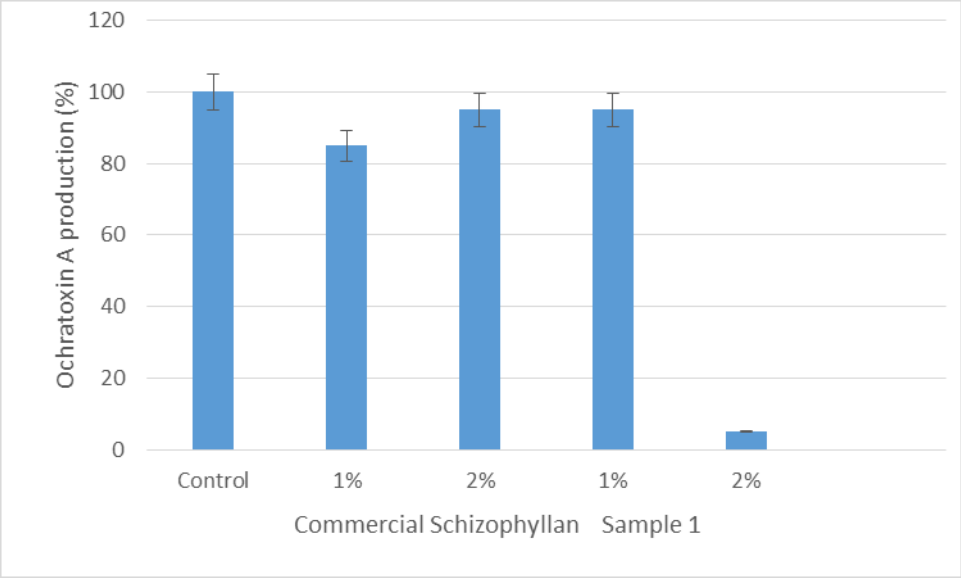


Figure 4. Comparison of the commercial Schizophyllan and partially purified *Schizophyllum commune* exo-polysaccharide (Sample 1) on inhibition of OTA synthesis.

The results show that commercial Schizophyllan showed a significantly lower impact on the inhibition of ochratoxin A synthesis than the semipurified exo-polysaccharide *S. commune* (Sample 1). Sample 1 showed the greatest effect on the inhibition (up to 95%) of OTA synthesis at a concentration of 2% (w/v).

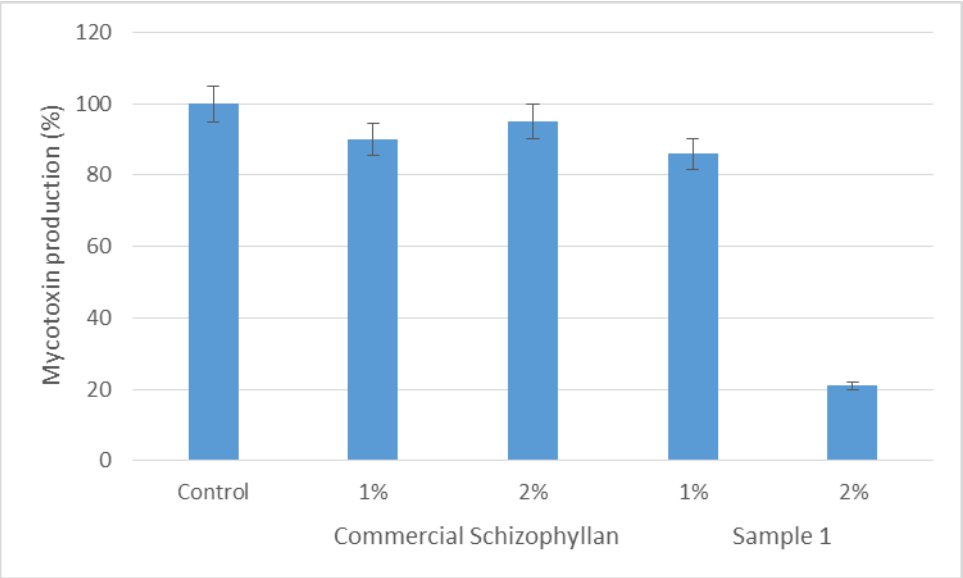


Figure 5. Comparison of the commercial Schizophyllan and partially purified *Schizophyllum commune* exo-polysaccharide (Sample 1) on inhibition of aflatoxin B1synthesis.

The results show that commercial Schizophyllan showed no significant effect on the control of mycotoxin synthesis at concentrations of 1% and 2%. Although, Sample 1 concentration of 2% (w/v) showed a significant effect on the inhibition of AFB_1 synthesis up to 80%.

4.3.4. Assay of *S. commune* rough filtrate on ochratoxin A synthesis and ochratoxin A synthesis within the time interval (24-72h)

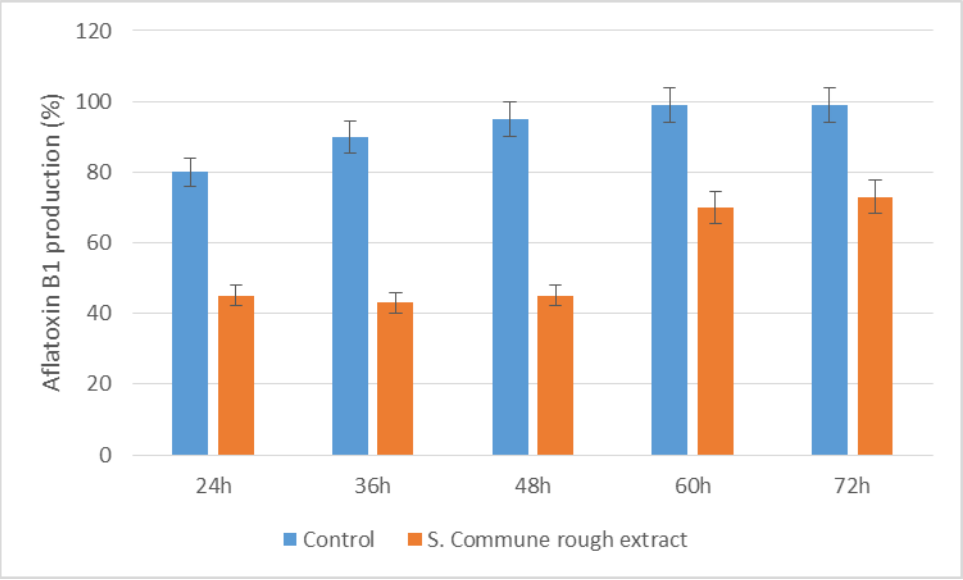


Figure 6. Effect of the lyophilised crude filtrates of *S. commune* (2% w/v), added to the PDB media inoculated with *A. flavus* of aflatoxin B1 formation in the culture filtrates. The samples

were incubated at 30° C in dark conditions and harvested every 12 hours in a period 24-72 hours. Although, we can confirm the influence of the rough extract of *S. commune* on aflatoxin synthesis between 24 and 48 h (which ranges from 55-57%), aflatoxin inhibition declines over a longer period (more than 72 h of incubation). The data represent the mean of ±SEM from n=3 samples for each time point from three separate experiments.

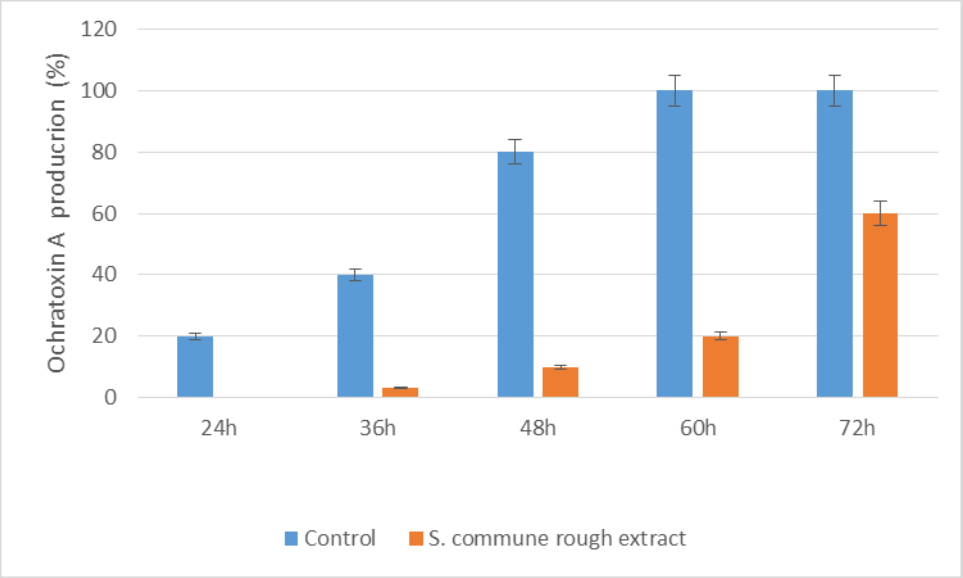
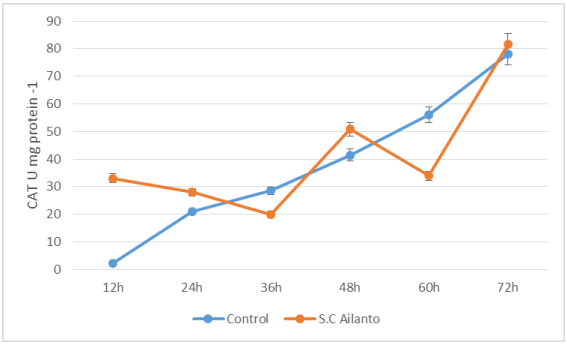


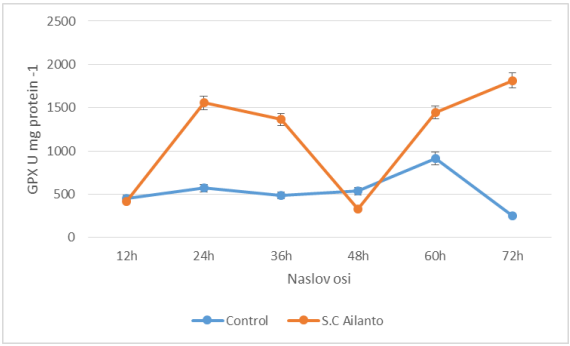
Figure 7. The influence of the lyophilized raw substrate of *S. commune* showed a significant effect on the inhibition of ochtatoxin A synthesis in the range of 97% -40% within a time period of 36h-72h. The data represent the mean of ±SEM from n=3 samples for each time point from three separate experiments.

4.3.5 Anti-oxidant enzymes activities in the mycelium of *A. flavus* and *A. carbonarius* in the presence and absence of lyophylised rough filtrate of *S. commune*

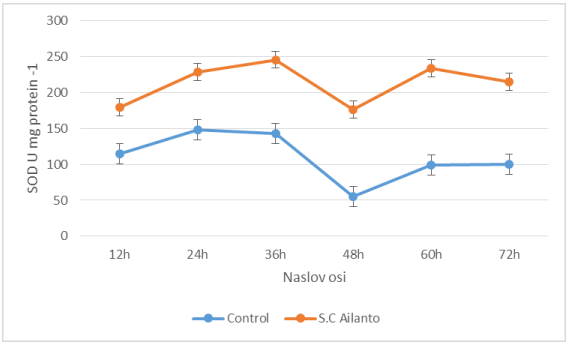
The activities of superoxide dismutase (SOD) pH7.8, catalase (CAT), and glutathione peroxidase(GPX) were analyzed in (50 mg) mycelia of *A. flavus* and *A. carbonarius* treated and non-treated with (2% w/v) of lyophylised rough filtrate of *S. commune* as previously described at Reverberi et al. (2008). The antioxidant activities were reported as such, as well as the sum of absolute values obtained by dividing the activity of SOD, CATor GPX per each time interval in the treated samples with the respective values in the untreated control.



a) CAT



b) GPX

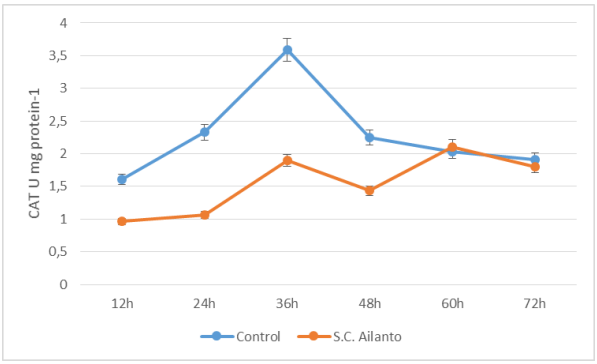


c) SOD

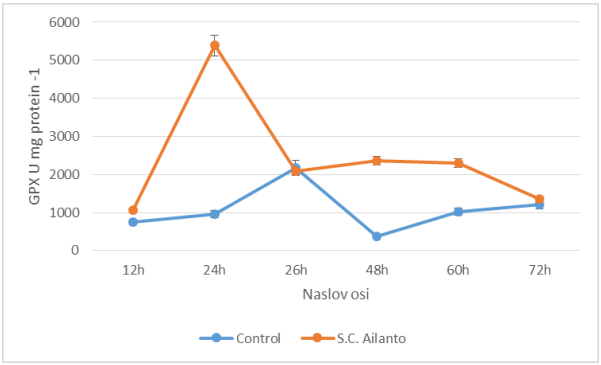
Figure 8. (a, b, c). The activity of antioxidant enzymes SOD, CAT and GPX (during the time course) in the mycelium of *A. flavus* treated and not treated with lyophilised raw filtrates of *S. commune*

SOD activity (Fig. 8 c) was significantly ($p < 0.001$ at 36h) stimulated by the addition of lyophilised filtrates in the substrate in comparison to control, and a peak was evident at 36 h of incubation. Even if a decrease appeared after this time, the activity of the SOD enzymes remained higher than that in control up to 72 h of incubation. CAT (Fig. 8 a) activity in the presence of lyophilised raw filtrates of *S. commune* was not significantly different compared to

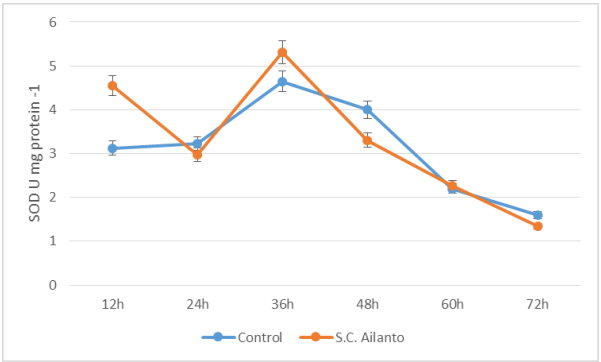
those detected in the control. GPX (Fig. 8 b) was significantly stimulated by the presence of lyophilised filtrates with a hihgest peak during the 72 h of incubation.



a) CAT



b) GPX



c) SOD

Figure 9. (a, b, c). The activity of antioxidant enzymes SOD, CAT and GPX (during the time course) in the mycelium of *A. carbonarius* treated and not treated with lyophilised raw filtrates of *S. commune.*

CAT activity was significantly stimulated by the addition of the lyophilised filtrates of *S. commune* in the substrate in comparison to control (Fig. 9 a) with a highest peak at 36 h of incubation. After 36 h the CAT enzyme has decreased, and at 72 h of incubation reached control level. SOD activity was not significantly stimulated by the addition of the lyophilised filtrates of *S. commune* in the substrate in comparison to control (Fig. 9 c). GPX was significantly stimulated by the presence of lyophilised filtrates with a highest peak at 24 h of incubation (Fig. 9 b).

Statistics:

Datasets were pooled and compared using one- or two-way ANOVA followed by Student t-test and the differences were considered significant when the p-value was <0.05 or in other cases <0.001.

4.4 Discussion

In this study, we demonstrated the influence of three different extracts of exo-polysaccharide from mushroom *S. commune* on inhibition of the synthesis of mycotoxins in *A. flavus* and *A. carbonarius*. The results showed that commercial Schizophyllan had no significant effect on the inhibition of ochratoxin and aflatoxin synthesis. The best inhibiting results on the aflatoxin and ochratoxin A production shows the semi-purified extract of *S. commune* at concentration of 2% (w/v). This effect could probably be due to its viscosity and different compounds present in the extracts with different inhibiting strategies. This polysaccharide needs further purification, the components should be separated and individually tested for mycotoxin inhibition. The higher efficiency of polysaccharides at higher concentration (2%) on the inhibition of mycotoxins may indicate that the activity of the polysaccharide is due to size of the polysaccharide and also its three-dimensional structure. It is known from the literature that the activity of these polysaccharides is size dependent, and that high molecular weight glucans appear to more effective than those of low molecular weight (Jong et al., 1991; Sakagami et al., 1991).

The application of these extracts to control aflatoxins in seeds maize and wheat seeds allows to consider its application without further purification steps. This is very interesting for possible use on a large scale because of its lower costs in comparison with purified fractions. *T. versicolor* isolates studied in this research could result as possible agents in biological control

or useful components in the integrated strategies against mycotoxin producing fungi in food and feed. Moreover the addition of the assayed mushrooms could contribute to enhance the nutritional value of feed; in fact they contain nutritive and active compounds. This enhances the value of the treated commodities beyond the conventional nutritional effects, so the feed obtained in consequence of the addition of the selected basidiomycetes could be considered a functional feed (Xu, 2001).

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Chapter 5. Final discussion and conclusions

Mycotoxins are becoming an increasingly important topic in both human and animal fields (Rodrigues and Naehrer, 2012). Different strategies for control and decontamination of mycotoxins in food and feed have been proposed, but none of them has solved the problem of mycotoxin contamination (Varga and Tóth, 2005). It is known that higher mushrooms secrete exo-polysaccharides and other compounds into their environment which are known to act as biological response modifyer (BRM) (Kidd, 2000; Kozarski et al., 2013; Zhang et al., 2016). These molecules achieve their immunostimulating effect indirectly, through activation of various defensive immune responses (Jong and Birmingham, 1992; Hobbs, 1995; Mizuno, 1995; 1999, Wasser and Weiss, 1999). Previous research has demonstrated that higher fungal polysaccharides are able to regulate the oxidants/antioxidants imbalance within the cell, which can significantly reduce the synthesis of mycotoxins such as AFB1 and OTA (Zjalic et al., 2006; Scarpari, et al., 2014; 2015; 2017; Reverberi et al., 2005; 2011) . Moreover, the exo-polysaccharides extracted from lignolytic basidiomycetes such as *L. edodes* and *T. verticolor* have been shown to have a positive effect on the inhibition of mycotoxin biosynthesis in *A. parasiticus*, *A. flavus* and *A. carbonarius* (Scarpari et al., 2014; 2015; 2017; Zjalic et al., 2006; Reverberi et al., 2008; 2011). Therefore, control of mycotoxins with mushroom polysaccharides may present a possible eco-friendly tool with a minor impact on the environment (Zjalic et al., 2006). One of the most studied fungi that affects mycotoxin synthesis is *T. versicolor*. It is known that crude lyophilised culture media and mycelia extracts from *T. versicolor* showed the long-lasting ability to inhibit the synthesis of aflatoxins both in vitro and in vivo (Zjalic et al., 2006; Scarpari et al., 2015; Reverberi et al., 2008). The active component of the extracts was found to be a polysaccharide. Exo-polysaccharide from *T. versicolor* culture filtrate was isolated, part of its primary structure was characterised and previously registred as Tramesan (Scarpari et al., 2017). These studies showed that Tramesan was able to act as a pro-antioxidant in different organisms. By enhancing the "natural" antioxidant defences of the "hosts", Tramesan could represent a useful tool for controlling the synthesis of several mycotoxins simultaneously (Zjalic et al., 2006; Reverberi et al., 2011). Although, the positive properties of Tramesan have been proven, due to changing environmental conditions, random mutations may occur, which could alter the structure of the active polysaccharide moiety. As the structure of Tramesan is only partially characterized we attempted to investigate the smallest fraction of oligosaccharide active in the inhibition of AFB_1 and OTA. synthesis. The purified

exopolysaccharide of *T. versicolor* was partially hydrolyzed and separated by column chromatography into fractions from disaccharides to heptasaccharides. Each fraction was individually tested (100μM and 200μM concentrations), for AFB_1 inhibition in *A. flavus* and OTA in *A. carbonarius*. Fragments smaller than 7 units showed no significant effect on mycotoxin inhibition, whereas heptasaccharides showed inhibitory activity up to 90% (100 μM) and 55% (200 μM) in *A. flavus*, and up to 81% (100 μM) and 78% (200 μM) in *A. carbonarius.* Since all of the oligosaccharides are composed of a mixture of two oligosaccharides of the same length but with different composition, at present it is not possible to determine which one, of the two fractions, is active, or if both have to be present. Besides, synergistic or antagonistic effects of the two different heptamers from the mixture on AFB_1 synthesis cannot be excluded. In future research, it will be necessary to separate the heptamers in order to characterize the exact structure of the active moiety and to avoid the potential interaction of the two different heptamers. Specifically, for di, three, penta and hexasaccharides, a higher concentration of oligosaccharides has shown to promote aflatoxin synthesis. Also, the production of OTA was enhanced by the presence of tetramers and pentamers. This could be explained by the use of oligosaccharides as carbohydrate supplementary source, which in turn favours mycotoxin synthesis (Fanelli et al., 1981). However, not always oligosaccharide concentration and aflatoxin inhibition are directly correlated. The results obtained using tetrasaccharide fractions showed significant enhancement in AFB_1 (80%) and OTA (34%) synthesis, within the concentration of 100μM. This phenomenon has already reported in previous studies on mycotoxin inhibition (Fanelli et al., 1980). In fact, these may be dose dependent actions, in which we have an increased effect of low concentration of sugar on aflatoxin synthesis. In addition, heptasaccharides, that is the largest fragments of the polysaccharide investigated, have shown the greatest influence on the control of mycotoxin synthesis. From these results, we can assume that the active polysaccharide fraction is greater than seven units, and that a lower concentration of heptaccharides has a better effect on AFB_1 and OTA inhibition (on account of the possible degradation of oligosaccharides, which then stimulates mycotoxin production). These results are also consistent with previous research on the correlation between polysaccharide size and their activity in mycotoxin inhibition (Scarpari et al., 2015). Therefore, we might assume that the size of the oligosaccharides is important for the binding to a specific receptor in the cell wall, and that longer oligosaccharides are, they are more likely to bind and elicit a cell response. Since it is proven that larger parts of oligosaccharides are active in mycotoxin control, the characterization of heptamers is ongoing and the study of the active structure is underway. Also, biotechnological methods are required in order to separate the

mixture of heptamers to determine which component is potentially responsible on aflatoxin and ochratoxin A inhibition. In addition, in the future it will be necessary to produce octa and nanosaccharides and to test their biological activity in controlling the mycotoxin production. In order to find a structure similar to Tramesan that has the effect of inhibiting multiple mycotoxin synthesis simultaneously, 42 different isolates of different mushrooms were investigated. For the mycotoxin inhibition assays, rough isolates of individual isolates at a concentration of 2% were used. The results demonstrated that different isolates of the same species of mushrooms have different effects on inhibition of AFB_1 and OTA synthesis. These variations could depend on genetic structure of the strain and its capacity to produce inhibiting compound(s), also there is a possibility that active compounds could be produced under different environmental conditions (temperature, oxygenation etc). The inhibition of one of the two mycotoxins, or a big differences in inhibition rates between them could result from the presence of some antioxidant compound (e.g., phenolic compound) which is more active (or appropriate) in inhibition of one of the two mycotoxins. The variability in inhibition rates among the different isolates of the same species is consistent with the results obtained by Reverberi et al. (2005) and Zjalic et al. (2006). The authors have also demonstrated that in cases of mushrooms *Trametes versicolor* and *Lentinula edodes* the inhibitory compound was a polysaccharide, which acted through enhancement and of the antioxidative response in the cells of mycotoxigenic species such as *A. parasiticus.* The proteins that were involved in this enhancement were Yap protein in *A. parasiticus* and its homologue Yaop in *A. ochraceus*. Given the similar mechanism of action a large number of mushroom exopolysaccharides tested for AFB_1 and OTA inhibition were expected to be active in inhibition of both mycotoxins. Among all isolated mushroom polysaccharides, the only lyophilized rough filtrate that gave a high inhibition of both mycotoxins (over 90%) were those of *T. versicolor* strain TV117 and *S. commune* strain S.C Ailanto. Since it is known that TV 117 produces a polysaccharide named Tramesan and that it is active in aflatoxin inhibition, therefore the research focused on the metabolite of mushroom *Schizophyllum commune*. *S. commune* is known to secreted polysaccharide schizophyllan in submerged culture medium, who is able to act as a biological response modifier (BRM) and non-specific stimulator of the immune system (Zhang et al, 2013). The comparison between the inhibition of mycotoxin synthesis by means of commercial β-glucan Schizophyllan and *S. Commune* (S.C. Ailanto) rough extract, shows a higher inhibition rate for unpurified S. C. Ailanto extract. This effect could probably be due to different compounds present in the unpurified extract, (such as proteins), with different inhibiting strategies. Furthermore, S. C. Ailanto extract exhibited the highest inhibition rate of both

mycotoxins at a concentration of 2% w/v, leading to the conclusion that some larger oligosaccharide or perhaps polysaccharide is active in blocking mycotoxin synthesis, which requires future research. It could be supposed that in this case the active compound is polysaccharide. It is confirmed by the experiment with schizophyllan, a polysaccharide produced by *S. commune* and produced commercialy. This polysaccharide showed a possibility to control the synhesis of both mycotoxins up to 3 days, a time in which at the experimental conditions was reached the plateau. The lower inhibiting rate for both mycotoxins, compared to rough filtrate of S. C Ailanto, indicates that Schizophyllan might be involved in inhibition but other molecules are needed to obtain higher efficiency in inhibition. Schyzophillan is a large polysaccharide of several hundreds of kDa, and its activity depends on its configuration (Kidd, 2000). Maybe other molecules produced by mushroom are necessary to obtain the wright configuration that could be active in aflatoxin and ochratoxin A inhibition. Anyhow, further research on involvement of Schizophyllan in inhibition of the synthesis of aflatoxins and ochratoxin A are needed, to clarify if this polysaccharide, or its parts, is a part of this inhibition and the role and nature of other *S. commune* metabolites involved. In this research we also, demonstrated that the presence of liophylised raw extracts of the cultures of *S. commune* enhanced and anticipated the activity of antioxidant enzymes in cells of both *A. flavus* and *A. carbonarius*. In both cases the biggest enhacment was observed for the activity of enzyme GPX . Similar results were observed previously whan the liophilyzed filtrates of *Lentinula edodes* and *T. versicolor* cultures were used for control of the sinthesys of aflatoxins in A parasitcus (Reverberi et al., 2005, Zjalic et al., 2006). In addition, it could be suggested that, besides showing the antioxidant activity per se, the S.C. Ailanto lyophilized raw filtrates influence the cascade of signals which allows mycotoxin biosynthesis, even if we can not indicate at which step of the pathway this event occurs. Nevertheless, it could be hypothesised that β-glucans and other compounds contained in raw filtrate of S.C. Ailanto, are able to inhibit mycotoxin synthesis in *A. flavus* and *A. carbonarius* by enhancing the internal antioxidant system. Anyhow, future research is required to determine the specific part of the polysaccharide active in the inhibition of AF and OTA, so that it can be synthetically produced and used for large scale application is agriculture. In particular, the discovery of the very active part of the polysaccharide would be especially relevant, from an economic point of view, as its synthesis would be more cost-effective in comparison with commercial Schizophyllan. Moreover, fungal extracts could represent a promising tool in myco-toxin control compared to chemicals or natural products as plant extracts (Mahoney and Molyneux, 2004), because of their low toxicity when released in the environment and for their easy growing conditions and extraction

procedures. In conclusion, the *S. commune* isolate studied in this research could be regarded as a possible agent in biological control, or as a useful component among the integrated strategies against mycotoxin producing fungi in food and feed products. Moreover, future research on Tramesan oligosaccharides could present a new environmentally friendly tool for controlling ochratoxin A and aflatoxin contamination. The possible synthetic production of the active part of Tramesan could be economically acceptable, and thus would allowed the use of this compound on large scale in agriculture.

Novel findings in this research:

- This research indicates some novel insights on the active structures of Tramesan oligosaccharides (i.e., heptasaccharides) in controlling mycotoxin production , which could lead to the synthetic production of the active moiety and then in its widespread use in agriculture.
- *S. commune* isolate studied in this research could be regarded as a possible agent in biological control, or as a useful component among the integrated strategies against mycotoxin producing fungi in food and feed products.

Future directions:

- The structure of the heptasaccharides should be further investigated. Moreover, a research on longer polysaccharides shold be performed to establish the optimal lenght and composition of polysaccharide moiety active in mycotoxin inhibition.

- The role of noncassharidic mushroom compounds as enhancer of the mycotoxin inhibition should be further investigated.

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