INSTITUTO POLITÉCNICO DE BRAGANÇA Escola Superior Agrária

Effect of commercial starter cultures and native yeasts on Ochratoxin A production by *Aspergillus westerdijkiae* and *Penicillium nordicum* in meat products

SANA MEFTAH

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Supervised by
Paula Rodrigues
Teresa Dias
Salwa Abid

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Success consists of going from failure to failure without loss of enthusiasm

Winston Churchill

ABSTRACT

Processed meat products are of worldwide importance and, because of their intrinsic factors as well as the processing methods, they are highly prone to fungal and mycotoxin contamination. Ochratoxin A (OTA) is the most significant mycotoxin in processed meat products. *Penicillium nordicum* is considered to be responsible for OTA contamination of meat products, as it is highly adapted to salt and protein-rich matrices and is moderately psycrotrophic. However, another OTA-producing fungus, *Aspergillus westerdijkiae,* adapted to carbon-rich matrices such as cereals and coffee beans, has been recently associated with high levels of OTA in meat products.
Several Lactic Acid Bacteria (LAB) and yeasts have been tested as biocontrol agents against *P. nordicum* growth and OTA production in meat products, with promising results, but none of the studies have considered *A. westerdijkiae*. The aim of this work was to evaluate *in vitro* the effect of a commercial starter culture used in sausage fermentation and four yeasts isolated from dry-cured sausage on these two OTA-producing fungi, both in terms of fungal growth and of OTA production, using different meat-based culture media as model systems. The mechanisms underlying the observed effect were also studied.

For this purpose, *C. krusei, C. zeylanoides, R. mucilaginosa, R. glutinis,* a mix of these yeasts and the starter culture were co-inoculated with *P. nordicum* and *A. westerdijkiae* in industrial sausage, traditional sausage, and ham-based media, under conditions of water activity, salt concentration and temperature that mimic real conditions at beginning and end of sausage curing process. Fungal growth was determined by measuring colony diameter, and OTA production was quantified by HPLC-FLD after extraction with methanol.

Yeasts where found to inhibit significantly the growth of both fungi. *P. nordicum* was unable to produce detectable OTA in both sausage-based media under any condition. In ham, yeasts reduced OTA production, while the starter culture significantly increased it. Unexpectedly, OTA production by *A. westerdijkiae* was significantly stimulated in all media tested by all microorganisms. Matrix has a significant effect on OTA production by *P. nordicum*, but not by *A. westerdijkiae*, for which only temperature showed to have effect. By testing the mechanisms of action by which starter culture and *C. zeylanoides* influenced fungal responses, we were able to determine that direct contact and simultaneous growth of test organisms were the mechanisms more significantly involved in the responses.

In conclusion, ochratoxigenic fungi do not all respond to antagonistic microorganisms in the same way. The use of biocontrol agents with the intent of reducing fungal growth and mycotoxin production by one fungus can have unexpected effects on others, thus leading to unforeseen safety problems. Further experiments are recommended to properly understand the reasons behind the different effects of microorganisms, to ensure their safe as biocontrol agents.

Keywords: Mycotoxins, *Aspergillus westerdijkiae*, *Penicillium nordicum*, HPLC-FLD, yeast, starter culture, biocontrol, food safety, pork meat

RESUMO

Os produtos processados de origem animal assumem importância mundial e, devido às suas características intrínsecas e métodos de processamento, são geralmente muito expostos a contaminação por fungos e micotoxinas. Ocratoxina A (OTA) é uma das micotoxinas de maior relevância nestes produtos alimentares, sendo *Penicillium nordicum* o fungo considerado responsável pela contaminação de produtos cárneos, devido à sua elevada adaptabilidade a matrizes ricas em proteína e sal. No entanto, o fungo *Aspergillus westerdijkiae*, reconhecidamente adaptado a matrizes vegetais ricas em carbono, foi recentemente associado a elevados níveis de OTA em presunto.
Várias Bactérias do Ácido Láctico e leveduras foram anteriormente testadas como agentes de biocontrolo contra *P. nordicum*, mas nenhum destes estudos considerou o efeito desses microrganismos em *A. westerdijkiae* em matrizes cárneas. O objetivo do presente estudo foi avaliar *in vitro* o efeito de uma cultura iniciadora (*starter*) comercial usada na fermentação de chouriço e de quatro leveduras previamente isoladas de chouriço português no crescimento e produção de OTA por estes dois fungos, usando meios de cultura de produtos cárneos com modelo. Os mecanismos de ação destes microrganismos teste sobre os fungos foram também analisados.
C. krusei, *C. zeylanoides*, *R. mucilaginosa*, *R. glutinis*, a mistura destas leveduras e a cultura iniciadora foram co-inoculadas com *P. nordicum* e *A. westerdijkiae* em meio de chouriço industrial, chouriço tradicional e presunto, sob condições de atividade de água, concentração de sal e temperatura que refletiam as condições de cura destes produtos. O crescimento dos fungos e a produção de OTA nas várias condições testadas foram avaliados por medição do crescimento da colónia e por quantificação por HPLC-FLD, respetivamente.

As leveduras reduziram significativamente o crescimento dos fungos. *P. nordicum* apenas produziu OTA detetável em presunto. Neste meio, as leveduras inibiram a produção de OTA por *P. nordicum*, enquanto a *starter* estimulou de forma significativa a sua produção. Inesperadamente, foi também observada a estimulação significativa da produção de OTA por *A. westerdijkiae* por todas as culturas testadas em todos os meios de cultura. O efeito de matriz foi significativo para *P. nordicum* mas não para *A. westerdijkiae*, para o qual apenas a temperatura teve efeito significativo.

O efeito das culturas testadas no crescimento e produção de OTA pelos fungos parece ser principalmente resultado do contacto direto e crescimento simultâneo do microrgnismo teste e fungo.

Em conclusão, os fungos ocratoxigénicos parecem não responder de forma igual aos microrganismos antagónicos. O uso de agentes de biocontrolo com o objetivo de reduzir o crescimento e produção de micotoxinas por determinado fungo pode ter efeitos indesejados sobre outros fungos, conduzindo a perigos alimentares imprevistos. É assim recomendado o desenvolvimento de mais estudos que permitam compreender as razões por detrás dos efeitos dos microrganismos controlo sobre os fungos, de forma a assegurar a segurança na sua utilização como agentes de biocontrolo.

Palavras-chave: Micotoxinas, *Aspergillus westerdijkiae*, *Penicillium nordicum*, HPLC-FLD, levedura, cultura iniciadora, biocontrolo, segurança alimentar, porco

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1. INTRODUCTION

1.1 Framework

Mycotoxins are natural substances produced as secondary metabolites of several filamentous fungi. They have worldwide distribution and affect a significant part of food and feed products. Mycotoxins pose a health risk to humans and animals due to their harmful biological properties. They also have a very wide economic impact resulting from health and veterinary care costs, reduction in livestock production, investment in research to reduce risks of mycotoxin problems (Zain, 2011).

Processed meat products such as dry-cured ham, fermented sausage and others are foods of major importance in several European countries, both nutritionally and economically. However, due to their characteristics, they are highly exposed to mycotoxin producing fungi.

Ochratoxin A (OTA) is the most significant mycotoxin found in processed meat products. *Penicillium nordicum* is considered to be responsible for OTA contamination of these products, as it is strongly adapted to salt and protein-rich matrices and is moderately psychrotrophic. However, another OTA-producing fungus, *Aspergillus westerdijkiae*, associated with carbon-rich matrices such as cereals and coffee beans, has been recently associated with high levels of OTA in meat products.

Because of its relevance, many countries have set limits on OTA levels in food, and it mandatory to develop efficient strategies to avoid it from entering the food chain. One of the major problems with OTA, as with mycotoxins in general, is its stability thus making it highly challenging to eliminate it from food products (Boudra et al., 1995). For this reason, one of the most promising strategies under study is to prevent its accumulation by creating the best conditions to inhibit OTA production.

Several Lactic Acid Bacteria (LAB) and yeasts have been tested as biocontrol agents against *P. nordicum* growth and OTA production in meat products, with promising results, but none of the studies have considered the effect of these microorganisms on *A. westerdijkiae*.

1.2. Objectives

At an initial stage of this work, the intended aims of this work were thus to study, in vitro, the effect of a commercial starter culture and of several native yeasts on *P. nordicum* and *A. westerdijkiae* growth and OTA production ability in a sausage-based medium, and to study the possibility of developing a biocontrol agent to be used as starter culture with antifungal activity. However, given some unexpected results obtained in the preliminary tests, we decided to test also the matrix effect, by testing those effects on two types of sausage and also on ham, for which we already had available data for comparison. Finally, the last aim of the work was to determine the mechanisms underlying the observed effects of yeasts and starter culture on fungi.

2. LITERATURE REVIEW

2.1 Mycotoxins in food: a serious health problem

Food is an essential part of everyone´s life, however, it can also be a serious danger to human health. In fact, it is usually a matrix for fungal growth. Those contaminants result not only on modification of flavours, rotting and reduced food quality but also on toxins production (Rodrigues, 2010). On the other hand, some fungi are used in food production processes to improve food quality.

Toxins produced by fungi are named mycotoxins. They are produced as secondary metabolites which are not involved directly in growth or development of the producing fungus, and its role is not well defined. In 1960, aflatoxin produced by *Aspergillus flavus* in peanut meal was the cause of the death of approximately 100,000 turkeys in England (Wannop, 1961), and since that, mycotoxins are considered as a huge health problem. Aflatoxins (B1, B2, G1 and G2), ochratoxin A, patulin, trichothecenes, fumonisins (B1, B2 and B3) and zearalenone are some of the most important mycotoxins studied for food safety (CAST, 2003). *Aspergillus*, *Penicillium* and *Fusarium* are the most important mycotoxin producing fungi.

Mycotoxin production depends on the fungus, the matrix and the environmental conditions. The same fungus is able to produce more than one mycotoxin and the same mycotoxin can be produced by different fungi.

2.2 Meat products

Pork meat is considered as a principal dish for European citizens, as it provides essential components to the body. In fact, for each 100 g of cooked meat, it offers 25 g of protein, it ensures mostly unsaturated fat, vitamins (especially those of group B) and minerals like iron, calcium, phosphorus, sodium, potassium, chlorine, magnesium. (Porc et équilibre alimentaire, URL: http://www.leporc.com/qualite/nutrition.html, accessed 05/03/2016).

According to Eurostat (2015), the number of pork livestock in the European Union countries in 2014 was 148.31 million heads (2.13 million heads in Portugal), and in terms of meat production, there was an increase of 0.9 % from 2013 to 2014, reaching 22.1 million tons. Portugal participates with 360 thousand tons.

In Europe, transformed pig products like ham and dry sausage are very popular. In markets, a large variety of different products can be found with different manufacturing processes that originate products with special organoleptic characteristics and appearance according to the country. Manufactory uses different physical and chemical methods for meat processing technology such as cutting, mixing, salting, utilization of spices, fermentation, smoking, curing, drying, heat treatment, among others (Asefa et al, 2010; principles of meat processing technology, URL: http://www.fao.org/docrep/010/ai407e/AI407E04.htm, accessed 07/03/2016)

Salting is one of the important steps not only for the taste but also to increase water retention. Theoretically, 15% salt corresponds to 0.91 of water activity resulting unfavorable conditions for microorganism's growth, while in practice this amount of NaCl is very high for acceptable and healthy foods. The usual low amount of salt (1.5-3%) used during meat production needs a combination with other preservation methods like heat treatment or moisture reduction (Heinz and Hautzinger, 2007).

In Portugal, the most consumed products are dry-cured ham and dry-fermented sausage. In general, dry-cured ham is produced by the addition of a mixture of salt and nitrite on the whole legs of the pork and curing for a long period (that can go up to 48 months in Iberian type). The smoking and drying process depends to the manufactory but is usually established at 12-24 °C with the control of humidity and air speed (Comi et al, 2004; Asefa et al, 2011; Rodríguez et al, 2012).

Many meat products are eaten as raw meat such as "Chouriço" which is a popular traditional dry cured sausage in Portugal. A mixture of fat, pure meat, salt, red pepper is stuffed into natural pork casings, smoked at 18-24 ºC and dried at 9 ºC during 25 days (Alfaia et al, 2015). Dry-fermented sausage can have 20 % to 30 % of weight loss during the fermentation process, reaching water activity levels that can go below 0.90 (Toldrá, 2002).

The ripening of dry-fermented meat products is a very complex and dynamic process involving a wide range of biochemical reactions. Native microorganisms play a significant role in the maturation process influencing the sensorial characteristics of the product, and the composition of the microbial community evolves throughout the fermentation and ripening processes. The dynamics of fermentative flora change during the production process and it is affected by processing and environmental conditions (temperature, water activity, oxygen,

metabolites, additives) (Toldrá, 2008). Ninety-five percent of the microbiota on the surface during the first days of fermentation is composed by yeasts. The population of yeasts is influenced by the production, the degree of smoking, the use of spices and the diameter of the sausages, but it is generally composed of *Debaryomyces, Candida, Rhodotorula, Trichosporon* and *Cryptococcus* (Simoncini, et al, 2007; Asefa et al, 2009a). After two weeks, the microbial community is equally formed by yeasts and molds, and at the end of the process (4-8 weeks) molds are the dominant microorganisms.

Relative humidity, temperature and water activity strongly influence mold growth. The most isolated mold species in fermented sausages belong to the genera *Penicillium, Aspergillus, Mucor* and *Cladosporium* (Sørensen et al, 2008; Iacumin et al, 2009; Castellari et al, 2010).

Many researchers reported that Lactic Acid Bacteria (LAB) have the ability to preserve the product until 5 days at room temperature and one month in the refrigerator due to its ability to produce antibacterial compounds like organic acids, hydrogen peroxide, diacetyl and bacteriocins. LAB bacteriocins are able to inhibit many pathogenic and food spoilage bacteria growth without any negative effects on human health (Huot et al, 1996; Leroy et al, 2006; Nguyen et al, 2010). Moreover, they have an influence on flavor, texture and nutritional value of the products due to their acidification abilities (Leroy and De Vuyst, 2004; Leroy et al, 2006).

Coagulase-negative staphylococci (CNS) are also present in fermented sausages. The most frequently isolated species are *Staphylococcus xylosus, S. eqourum, S. saprophyticus* and *S. carnosus* (Iacumin et al, 2006; Kozachinski et al, 2008). Their major role is the production of lipolysis and proteolysis by-products like peptides, amino acids and volatile compounds, contributing to specific flavor and texture (Hughes et al, 2002; Toldrá, 2008; Casaburi et al, 2008).

In the case of traditional production, fermentation takes place by endogenous flora, while for industrial sausage commercial starter cultures are used as a mix of LAB, CNS, yeasts and/or molds (Toldrá, 2008). *Lactobacillus casei, Lactobacillus curvatus, Lactobacillus pentosus, Lactobacillus sakei, Lactobacillus rhamnosus, Pediococcus acidilactici* and *Pediococcus pentosaceus* are the major LAB that are used as starter cultures (Veskovic, 2010). The previously mentioned CNS species are also used as starter cultures.

Fungi being used as starter cultures are *Penicillium nalgiovense, Penicillium chrysogenum, Penicillium camemberti* or *Penicillium gladioli* (Toldrá, 2002). The main consequences of

molds metabolism are the increase of ammonia, reduction of lactic acid and proteolytic activity which are partially responsible for the sensorial characteristics of fermented sausages. On the other hand, yeasts consume the oxygen and produce catalase to decompose peroxide (Lucke and Hechelmann, 1987). *Debaryomyces hansenni* is the most used species in commercial starters (Toldrá, 2002).

2.3 Ochratoxin A

2.3.1 General considerations

OTA is one of the most important and toxic mycotoxins found naturally in the food chain. Several metabolites are related to OTA, such as ochratoxin B (OTB; the dechloro analog of OTA), ochratoxin C (OTC; its ethyl ester), but they are not considered toxigenic (Ringot et al, 2006). OTA is considered to be responsible for nephrotoxicity, hepatotoxicity, genotoxicity, immunotoxicity, neurotoxicity and teratogenicity, and can result from the consumption or direct contact of foods contaminated with OTA and the inhalation of spores from ochratoxigenic fungi (Ringot et al, 2006). The mechanisms behind these effects are an increase in oxidative stress, inhibition of protein synthesis, disruption of calcium homeostasis, inhibition of mitochondrial respiration and DNA damage (Ringot et al, 2006). The effects of OTA can increase by synergy with other mycotoxins (Bennet and Klich, 2003).

The discovery of OTA occurred in 1965 by Van der Merwe and coworkers (Van der Merwe et al, 1965) as a metabolite of *A. ochraceus*. From that time, OTA has been frequently found in cereal crops, coffee, cheese and meat products, usually associated with *P. verrucosum* and *P. nordicum* (Pitt and Hocking, 2009). Currently OTA production is associated also to several species of *Aspergillus* such as *A. niger, A carbonarius*, *A. steynii* and *A. westerdijkiae* (Gil-Serna et al, 2014, 2015).

2.3.2 Structure and physicochemical properties

Figure 2.1 represents the chemical structure of OTA. It is an organic anion, the 7-carboxy-5-chloro-8-hydroxy-3,4-dihydro-3-methylisocoumarin, linked to phenylalanine by an amide bond (Van der Merwe et al, 1965).

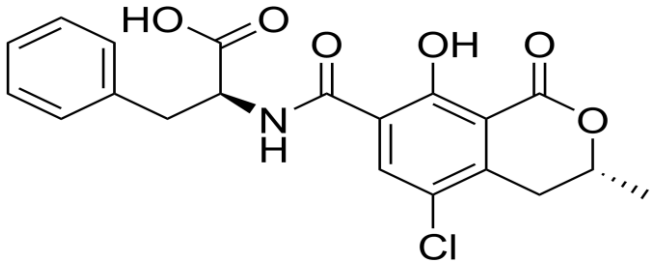


Figure 2.1 Chemical structure of ochratoxin A (OTA)

The molecular formula of OTA is $C_{20}H_{18}ClNO_6$, and it is a white crystalline with molar mass of 403.8 $g.mol^{-1}$ and a pKa value of 7.1. OTA is soluble in polar organic solvents (chloroform, acetone, ethanol) slightly soluble in water and insoluble in saturated hydrocarbons (El Khoury and Atoui, 2010). It has a maximum emission at 467 nm in 98% ethanol and 428 nm in absolute ethanol.

2.3.3 Mycotoxinogenesis

The biosynthetic pathway of OTA is not very clear. Ringot et al (2006) showed that phenylalanine originates from the shikimic acid pathway and dihydroisocoumarin (Otα) is originated from the polyketides pathway. OTA is then formed from these precursors by ochratoxin synthase which catalyses the sequence between OTα and phenylalanine **(Figure 2.2**).

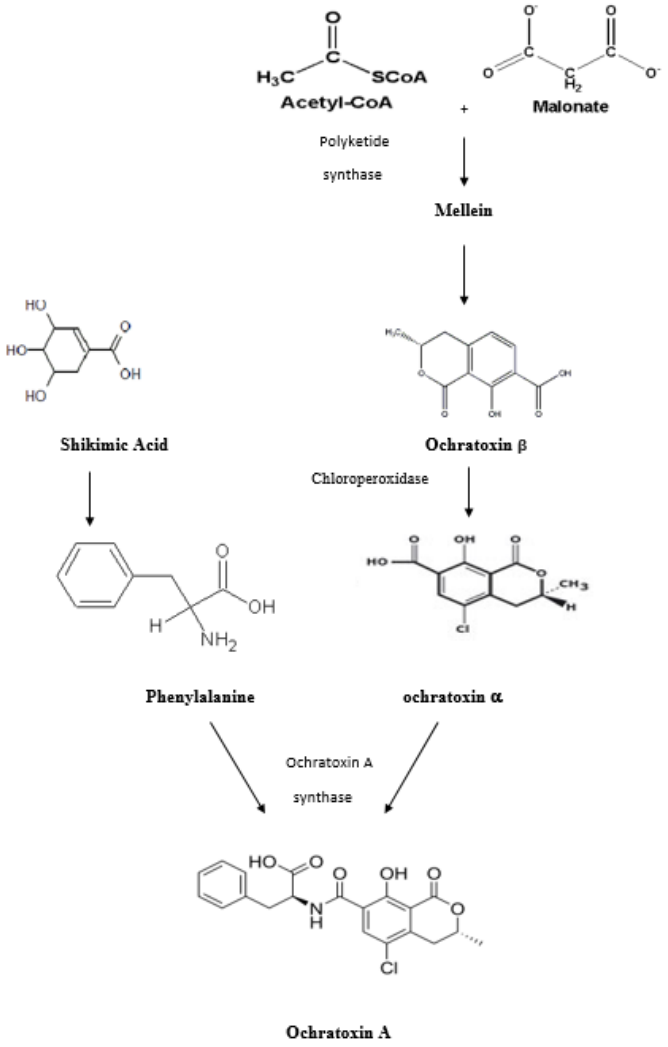


Figure 2.2 Biosynthesis of OTA (adapted from Ringot et al, 2006)

2.4 Ochratoxin A and ochratoxigenic fungi in meat products

2.4.1 OTA in meat products

Animal-derived products for human consumption may be contaminated with OTA if the animal has been fed with OTA contaminated feedstuff (Dall'Asta et al, 2010). Among farmed animals, pigs are known to be particularly sensitive to OTA accumulation.

OTA carry-over from fresh meat is a possible route of processed meat products contamination, since OTA is a moderately stable molecule that remains unaltered during most methods of food processing (e.g. heating and ripening), storage and preparation (Monaci et al, 2005; Schiavone et al, 2008). However, OTA contamination of processed meat products usually results from moulds growing during ripening. Several studies have reported OTA

contamination of processed meat products. An average of 0.53 μg/kg has been found on the surface of 76% of dry-cured ham samples with maximum level of 12.51 μg/kg (Dall´Asta, 2010). Sørensen et al (2010) analysed 22 retail Ham samples, and one of the most expensive type contained 56 μg/kg OTA, while 158 and 113 μg/kg were found in 2 frozen samples from the same type of product. Moreover, 50% of dry-cured Iberian ham samples showed a high OTA concentration going from 2 μg/kg to 160.9 μg/kg (Rodríguez et al, 2012). Moreover, between 3-18 μg/kg of OTA concentration has been found in Italian sausage (Lacumin et al, 2009).

The Scientific Committee for Food (SCF) and the Joint FAO/WHO Expert Committee on Food Additives (JECFA) have set allowable levels of OTA daily intake between 5 and 14 ng/kg bw/day. The European Commission (EC) and European Food Safety Authority (EFSA) consider that the contamination by meat products is insignificant and no limit values have been established (EFSA, 2006). On the other hand, Denmark, Italy, Romania, Slovakia and Estonia, which are typically strong consumers of pork meat and meat products imposed limits of OTA concentration that range from 1 to 10 μg/kg (Duarte et al, 2010a).

2.4.2 Ochratoxigenic fungi in meat products

Fungal growth and mycotoxin production only occur under favourable conditions, which vary for each species depending on adaptability. Food intrinsic parameters associated to extrinsic factors are responsible for the spectrum of contaminating and dominating mycobiota. This is mostly related to the physiology of fungi and their adaptation to the different matrices and environmental conditions (Rodrigues, 2010). In particular, it is the interaction between some or all of these factors that determines whether contamination increases and mycotoxins are produced. Interactions between available water and temperature are fundamental because they represent the two-dimensional niche in which fungi may be able to germinate, grow and actively compete for the allocation of the available resources. It is also generally well agreed that, in contrast to bacterial growth, water activity is the most significant factor controlling fungal growth (Rodrigues, 2010; Medina et al, 2015).

Several fungal genera are usually found during all meat production process (Comi et al, 2004). But the long drying period as well the environmental conditions, low temperature and high humidity in dry-cured ham and dry-fermented sausage favour the growth of *Penicillium*,

Aspergillus and *Eurotium* genera (Asefa et al, 2009b; Iacumin et al, 2009; Dall´Asta et al, 2010), some of which strongly toxigenic. *P. nordicum* and, to a lesser extent, *P. verrucosum* have been strongly associated to OTA production in dry-cured pork products (Bogs et al, 2006; Iacumin et al, 2009; Sonjak et al, 2011).

2.4.2.1 *Penicillium nordicum*

The genera *Penicillium* is known by their growth usually in shades of green, blue and sometimes white. Some produce penicillin, some can be opportunistic pathogens, and some produce mycotoxins. *Penicillium nordicum* (**Figure 2.3**) is notorious by its ability to produce OTA in protein and NaCl rich food matrices like processed meat products.

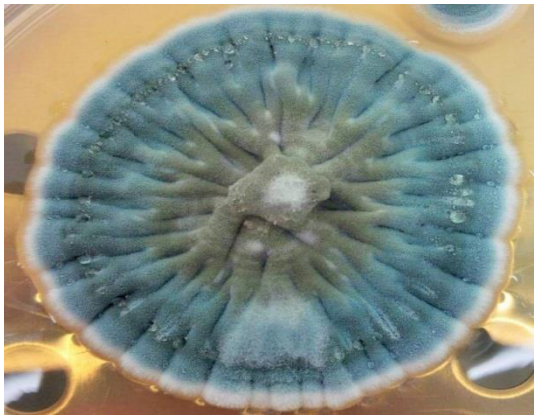


Figure 2.3 *Penicillium nordicum* on Czapek Yeast Autolysate (CYA) agar after 10 days of incubation at 25 ºC

This fungus prefers fresh and temperate climates. The optimal temperature of OTA production is 25 ºC, and the optimum pH is 6, with OTA being produced at high levels also at pH=5 and pH=8 (Geisen, 2004). It was isolated from ham, dry- fermented sausage (Battilani et al, 2007; Castellari et al, 2010; Rodríguez et al, 2012) responsible for high OTA concentrations in meat products. Rodríguez et al (2014) showed that *P. nordicum* grow and produce OTA better at 22% salt (0.87 aw) than at 10% (0.94 aw) in Ham.

2.4.2.2 *Aspergilus westerdijkiae*

Aspergillus usually grow on the surface of carbon rich substrates. They can be used in industrial processes but others are pathogenic and produce mycotoxins, like *A ochraceus*, *A. carbonarius*, *A. niger* and *A. westerdijkiae* (Machida and Gomi, 2010). *A. westerdijkiae* (**Figure 2.4**) was only recently described as a new species, and it was previously known as *A. ochraceus* (Frisvad et al, 2004). *A. westerdijkiae* is strongly associated to high OTA production in several foods such as grapes (Diaz et al, 2009) coffee (Leong et al, 2007; Morello et al, 2007; Gil-Serna et al, 2014), and cereals like barley (Mateo et al, 2011), wheat and maize (Duarte et al, 2010b).

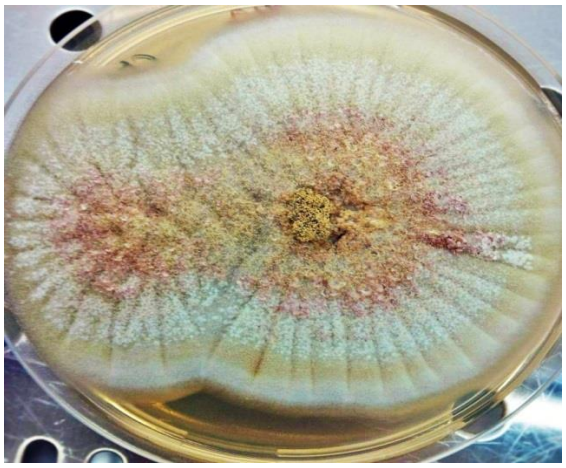


Figure 2.4 *A. westerdijkiae* on Czapek Yeast Autolysate (CYA) agar after 10 days of incubation at 25 ºC

For many years *A. westerdijkiae* was always linked to starch-rich substrates, it is considered important OTA-producer in green coffee beans (Leong et al, 2007). Moreover, Abdel-Hadi and Magan (2009) proved that it could produce OTA even at low temperature and water activity. Recently, researchers detected its presence also in meat products (Scaramuzza et al, 2015; Paula Rodrigues, pers. communic.). Optimal ecophysiological conditions of *A. westerdijkiae* growth and OTA production are 28 to 32 ºC and 0.93 to 0.99 water activity (Gil-Serna et al, 2014), but they vary with the matrix (Gil-Serna et al, 2015). Such ecophysiological conditions have been determined for vegetable matrices such as coffee, grapes, cereals and condiments, but have never been studied for meat products.

2.5 Mechanisms of OTA control in meat products

Because of its harmful effects, many researches have been done to avoid the accumulation of OTA in food products. This can be done either by inhibition of growth of ochratoxigenic fungi, inhibition of OTA production or degradation of already produced OTA. Theoretically, it is possible to find a strategy to inhibit the fungal growth or OTA production in meat by heating it enough or by adding high concentrations of preservative substrates, however, it is practically impossible because it may be unacceptable by the consumers or because of economic reasons.

Three different methods of mycotoxins prevention have been used in the last decades: i) physical: Var et al (2008) reported that activated carbon (AC) is an effective adsorbent of OTA and Refai et al (1996) found a total destroy of OTA by gamma irradiation; ii) chemical: Bellí (2006) demonstrated a significant reduction of fungal growth and OTA production treated with fungicides; and iii) microbiological: consists on the use of nonpathogenic microorganisms that exhibit diverse types of effects over OTA-producing fungi. The latter approach is considered the most interesting and promising strategy because, if proper biocontrol agents are used, it doesn't show the side effects on human health and on the characteristics of the product of the physical and chemical approaches.

Many studies over the years were done for the screening of yeasts, bacteria and fungi with antagonistic effect on OTA accumulation in food products, either by inhibition of fungal growth or by inhibition of OTA production. Several moulds, like black *Aspergilli* (Bejaoui et al, 2006) and *Penicillium adametzioides* (Ahmed et al, 2015) have been found to be able to degrade OTA or inhibit its production. Virgili et al (2012) demonstrated a significant reduction in growth of *P. nordicum* and OTA production in dry-cured ham by *Hyphopichia burtonii, Candida zeylanoides* and *Debaryomyces hansenii*, being the decrease in OTA production enhanced by the addition of NaCl. Simoncini et al (2014) reported an inhibition of *P. nordicum* growth and OTA production in dry-cured pork meat by *Derbaryomyces hansenii* and *Hyphopichia burtonii*. *A. westerdijkiae,* the popular OTA producer in coffee beans was controlled by *Pichia fermentans, Sporobolomyces roceus, Candida sp* and *Lactobacillus brevis* isolated from coffee fruits (Pereira et al, 2016).

3. MATERIALS AND METHODS

NOTE: This note intends to justify some experimental options assumed in the present study. The initial goal of our work was to determine the effect of some microorganisms on growth and OTA production by *P. nordicum* and *A. westerdijkiae* on industrial sausage. For that purpose, native yeasts and a commercial starter culture used in sausage fermentation and curing process were selected. Also, different salt concentrations/aw and temperatures were selected with the intention of reproducing sausage's curing conditions. However, after the first set of tests, unexpected results were obtained, which lead us to consider two possibilities: experimental errors or matrix effect. For that reason, two other matrices - traditional sausage and dry-cured ham - where introduced in the study. Traditional sausage was introduced with the objective of testing a similar matrix. Ham was used as matrix positive control, since it had been previously used in ecophysiological studies of both fungi, and we already had available data (Vipotnik et al, submitted).

3.1 Selection and identification of yeasts

3.1.1 Phenotypic identification of yeast isolates

In a previous work, several yeasts were randomly isolated throughout manufacture process of two traditional dry-fermented sausage production units, followed up through systematic sampling of raw meat, meat mixed with ingredients, macerated meat, smoked sausage and final product. From these, one hundred yeasts were grown on Potato Dextrose Agar (PDA, Liofilchem-ITALY) for 3 days at 28 ºC and preliminarily grouped according to their colony and cell morphology. From these groups, ten morphologically distinct yeasts were selected and biochemically identified by the rapid miniaturised system API 20 C AUX System (bioMérieux, France), following supplier instructions.

For this purpose, yeasts were grown on Potato Dextrose Agar and incubated at 28 °C for 3 days. A portion of a yeast colony was resuspended in NaCl (0,85%) to prepare a cell suspension with a turbidity equal to 2 McFarland. Then, 100 µL from this solution were transferred into 2 mL of API C medium. The suspension was used to fill each well of the strip, avoiding convex and concave surfaces, and the gallery was incubated at 29 °C for 72 h. Growth was determined

after 48 and 72 hours of incubation, by comparing the turbidity of the microtubes with that of control. A numerical profile of 7 digits was deduced and introduced in the database of API 20 C AUX (https://apiweb.biomerieux.com/) to identify the species.

Four of the identified species – *Rhodotorula glutinis*, *Candida krusei*, *Candida zeylanoides* and *Rhodotorula mucilaginosa* - were selected to test their effect on *P. nordicum* and *A. westerdijkiae* growth and OTA production on processed meat-based culture media. This selection was based on two parameters: yeasts should be non-pathogenic and should have been previously reported as adapted to meat products.

3.1.2 Molecular identification of selected yeasts

3.1.2.1 Genomic DNA extraction

Phenotypic identification of the selected yeasts was confirmed by sequencing of the D1/D2 region of 26S rRNA gene amplified by Polymerase Chain Reaction (PCR).

Yeasts were grown on Potato Dextrose Agar (PDA, Liofilchem-ITALY). After 3 days of incubation at 28 ºC, genomic DNA extraction was performed as described by Rodrigues et al. (2009). The biological material was transferred to a 1.5 mL tube containing 0.3 mL of Lysis Buffer (200 mM Tris-HCl pH 8.5; 250 mM NaCl; 25 mM EDTA; 0.5% [w/v] Sodium Dodecyl Sulphate) and approximately 0.5 g of sterile 0.4- to 0.6-mm diameter glass beads (Sigma, St. Louis, MO, USA), previously washed with nitric acid. The mixture was vortexed for 5 min at maximum speed. Polysaccharides and proteins were precipitated by adding 150 µL of cold 3 M sodium acetate, pH 5.5. This was gently mixed by inversion, placed at -20 ºC for 10 min and centrifuged twice at 14,000 x g for 10 min (4 ºC). Clean supernatant was then transferred to a new tube and precipitated with one volume of cold isopropanol (-20 ºC). This solution was gently mixed by inversion for a few minutes, incubated at -20 ºC for one hour and centrifuged at 14,000 x g for 10 min (4 ºC). DNA pellet was washed twice with 1.0 mL of cold 70 % ethanol, centrifuged at 6,000 x g for 7 min (4 ºC) and air dried. DNA was dissolved in 50 µL of ultra-pure water, and stored at -20 ºC until further use.

Quality and concentration of the obtained genomic DNA was determined by horizontal gel electrophoresis. Electrophoretic analysis was done on 1 % agarose gels with Tris-Acetate-EDTA buffer (TAE: 40 mM Tris-HCl; 40 mM acetic acid; 1.0 mM EDTA, pH 8.0) stained with

GelRed (VWR). Runs were made in TAE buffer, at constant voltage of 5 V/cm for approximately one hour. Five µL of genomic DNA and one µL of Orange Blue Loading Buffer (Promega) were loaded on the gel. DNA was visualised under UV light and images were obtained by the image analysis system Gel Doc^{TM} XR+ System (Biorad).

3.1.2.2 PCR amplification

PCR amplification of the D1/D2 region of 26S rRNA gene was achieved by using the primers NL1 (5'GCATATCAATAAGCGGAGGAAAAAG3') and NL4 (5'GGTCCGTGTTTCAAGACGG3'), as described by Kurtzman and Robnett (1998). PCR reactions were carried out in a thermal cycler BioRad Mycycler, in a final volume of 50 µL, containing 10 µL of 5x Go-Flexi Taq $MgCl_2$-free reaction Buffer (Promega), 1.5 mM $MgCl_2$, 1.25 U of Go-Flexi Taq polymerase (Promega), 200 µM of each dNTP (Bioron), and 2 µL of genomic DNA. PCR was carried out as follows: initial denaturation of DNA for 4 min at 95 °C; 34 cycles of denaturation at 95 ºC for 50 s, annealing at 55 °C for 50 s, and extension at 72 °C for 1 min; and a final extension cycle at 72 °C for 7 min.

PCR products of expected size *ca.* 600 bp were separated on a 1.2 % agarose/TAE gel stained with GelRed, and compared to a DNA size marker. Electrophoretic runs and image acquisition were as previously described.

3.1.2.3 PCR product purification

Before sequencing, PCR products were purified from excessive dNTPs and primers with the commercial kit GF-1 PCR CleanUp Kit (Vivantis), according to the instructions of the manufacturer. The volume of the PCR product was adjusted to 100 µL with sterile water and 500 µL of Buffer PCR were then added. After vortexing, the sample was transferred into a column assembled in a clean collection tube and centrifuged at 10.000 g for 1 min. The flow through was discarded and 750 µL of Wash Buffer were added. Wash Buffer was eliminated by two centrifugations at 10.000 g for 1 min. For DNA elution, the column was transferred to a clean eppendorf and 40 µL of sterile water were added. The PCR product was stored at 4 °C. The concentration of the purified PCR product was determined by electrophoresis as previously described and sent for sequencing.

3.1.2.4 Sequence analysis

Sequence analyses were performed on an ABI 3730xl DNA Analyzer (Applied Biosystems), by outsourcing. PCR products were sequenced in both directions, and a consensus sequence was created from the assembly of the forward and backward sequences using the package Sequencher 4.9 (Gene Codes, Ann Arbor Michigan). The consensus sequences were manually adjusted by chromatogram comparison and then aligned with the NCBI GenBank database (http://www.ncbi.nlm.nih.gov/) using the BLAST algorithm. The yeast isolates were identified by using a similarity threshold of 98 %.

3.2 Microorganisms and inocula preparation

3.2.1 Yeasts and starter culture

The selected yeasts - *Candida krusei* (Ck), *Candida zeylanoides* (Cz), *Rhodotorula glutinis* (Rg) and *Rhodotorula mucilaginosa* (Rm) - were sub-cultured from stock vials onto Potato Dextrose Agar (PDA, Liofilchem-ITALY) and incubated at 28 °C. For the preparation of the pre-inoculum, one colony from 3 day old cultures was suspended in Potato Dextrose Broth (PDB, Liofilchem-ITALY) and incubated at 28 °C for 24 h in a rotary shaker (120 rpm). Optical density of the suspension was determined by spectrophotometry at wave length 600 nm and an inoculum with approximately 10^5 cells/mL was prepared for all assays. Cell concentration was estimated by interpolation of absorbance values measured at 600 nm using the relationship OD600 = 1.0 corresponds to 3 x 10^7 cells/mL, as described by Day et al (2004).

Besides the native yeasts previously mentioned, a commercial freeze-dried starter culture (Texel®ELCE Br, Danisco) – composed by *Pediococcus pentosaceus*, *Lactobacillus sakei, Staphylococcus carnosus*, *Staphylococcus xylosus* and *Debaryomyces hansenii* – was also used. The freeze-dried starter culture (0.01% w/v) was inoculated in MRS (de Man, Rogosa, Sharpe) broth and incubated at 37 °C for 24 h. After that, 300 µL were used to inoculate 150 mL of each meat extract media.

3.2.2 Ochratoxigenic fungi

Two species of OTA-producing fungi, both isolated from dry-cured ham, were used: *Aspergillus westerdijkiae* strain 6B/131 (Paula Rodrigues, Polytechnic Institute of Bragança, Portugal) and *Penicillium nordicum* strain PN 44 (provided by Dr. Alicia Rodríguez, University of Extremadura, Spain) (**Figure 3.1**). Fungi were inoculated in Malt Extract Agar (MEA, Liofilchem-ITALY) and incubated for 10 days at 25 °C in the dark.

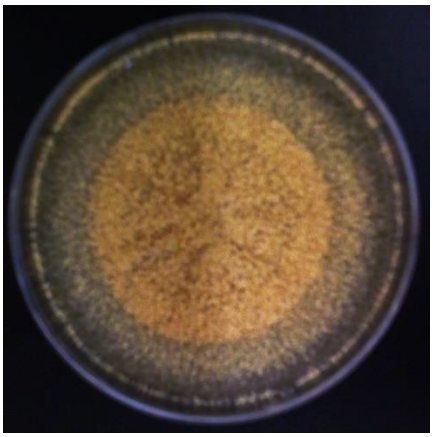
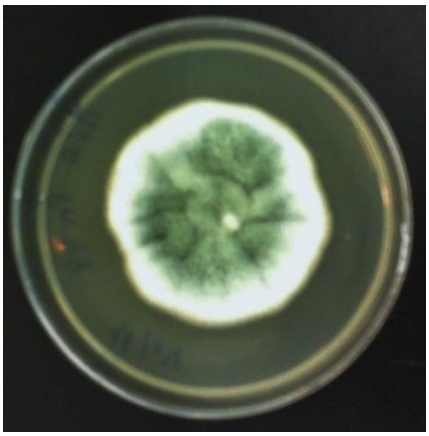
A  B 

Figure 3.1 A: *A. westerdijkiae* 6B/131 **B***: P. nordicum* PN 44; on MEA incubated for 10 days at 25

After incubation, 2 mL of sterilized water with 0.05% Tween 80 were added to the culture and spores were scrubbed to obtain a suspension. This spore suspension was adjusted to 10^7 spores/mL by counting cells with the aid of a Neubauer counting chamber.

3.3 Meat extract media preparation

Sausage based media were designed to mimic a_w and NaCl concentration usually found in Portuguese dry-fermented sausage (*chouriço*) in the two most significant steps of product production: final product and end of ripening (Gonzales-Barron et al, 2015). Ham based-medium characteristics in terms of water activity and salt concentration used in the present study are those for which best-fitting results were obtained in aprevious study (Vipotnk et al, submitted). So, the three matrices used in the study were industrial sausage (Ind), traditional sausage (Trad) and Ham (Ham).

For this purpose, all meat products were finely minced and lyophilized. Thirty grams of lyophilized meat product were boiled in 1000 mL of distilled water (3 % meat) during 30 minutes and filtered through cheese cloth. Meat extract was supplemented with 2 % or 10 % glycerol to reduce a_w and with NaCl at 1 % or 3 %, as described in **Table 3.1**, and volume was made up to 1000 mL. Media were solidified with 2 % of bacteriological agar (HiMedia), and were autoclaved at 121 ºC for 15 minutes.

pH was measured with a pH-meter (METTLER TOLEDO). Since all media showed pH ranging from 5.3 to 5.5, pH was not corrected. Water activity was measured using a water activity meter (Aqualab 4TE). Three independent values were obtained for each sample.

Table 3.1 Culture media characteristics

Matrix	NaCl	Glycerol	Water activity
Ind	1%	0	0.98
Ind	3%	10%	0.95
Trad	3%	10%	0.95
Ham	3%	2%	0.97

3.4 Effect of test microorganisms on fungal growth and OTA production

P. nordicum and *A. westerdijkiae* were co-inoculated with native yeasts and starter culture to determine the effect of the latter microorganisms on fungal growth and OTA production, under different conditions of temperature, water activity, concentration of NaCl and matrix. For the following assays, all co-inoculations were incubated for a period of 15 days. Fungal growth was determined at the end of incubation period by measurement of fungal colonies' diameter, in two directions at right angles. At the end of incubation period, all Petri dishes were submitted to OTA analysis as described below (**section 3.5**). Petri dishes without test microorganisms (fungi only) were used as control. Unless stated otherwise, all tests were run in duplicate.

3.4.1 Preliminary co-inoculation assays

In the manufacture of dry-fermented industrial sausage, during the ripening stage a gradual decrease in a_w and increase in NaCl concentration occurs, which leads to a change in the dynamics of microbial community. As a preliminary assay, the effect of each of the test microorganism on fungal growth and OTA production of *P. nordicum* and *A. westerdijkiae* was tested in Ind medium. For this assay, different levels of a_w and NaCl concentration where used as representative of Portuguese dry-fermented sausage at the beginning of ripening stage (1 % NaCl; a_w 0.98) and in the final product (3 % NaCl; a_w 0.95 adjusted with 10 % glycerol), as described by Gonzales-Barron et al (2015).

The Ind media were inoculated by incorporation of 10^5 cells/mL of the commercial starter culture, *R. glutinis, C. krusei, C. zeylanoides, R. mucilaginosa* and a mix of the 4 yeasts, and approximately 20 mL of the inoculated medium was poured into each petri dish. The mix of yeasts was used to stimulate the effect of yeast community usually present in the matrix. Two μL of fungal spore suspension (equivalent to 2 x 10^4 spores/spot) were spotted by equidistant three-point inoculation **(figure 3.2)** and incubated at 15 and 20 °C for 15 days. These temperatures mimic the ones used during the ripening stage. The different co-inoculations are described in **table 3.2**.

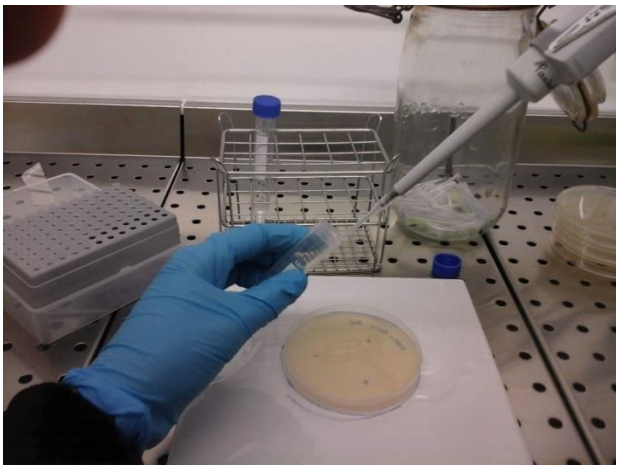


Figure 3.2 Co- inoculation with fungi

Table 3.2 Co-culture variables

Matrix	Temperature	Control							Test					
		Yeast/ Starter/Fungus							Yeast/Starter+Fungus					
1% NaCl	15 °C	Rm	Rg	Ck	Cz	Mix	St	Pn	Pn+Rm	Pn+Rg	Pn+Ck	Pn+Cz	Pn+Mix	Pn+St
	20 °C	Rm	Rg	Ck	Cz	Mix	St	Aw	Pn+Rm	Pn+Rg	Pn+Ck	Pn+Cz	Pn+Mix	Pn+St
	15 °C							Aw	Aw+Rm	Aw+Rg	Aw+Ck	Aw+Cz	Aw+Mix	Aw+St
	20 °C							Pn	Aw+Rm	Aw+Rg	Aw+Ck	Aw+Cz	Aw+Mix	Aw+St
3% NaCl	15 °C	Rm	Rg	Ck	Cz	Mix	St	Pn	Pn+Rm	Pn+Rg	Pn+Ck	Pn+Cz	Pn+Mix	Pn+St
	20 °C	Rm	Rg	Ck	Cz	Mix	St	Aw	Pn+Rm	Pn+Rg	Pn+Ck	Pn+Cz	Pn+Mix	Pn+St
	15 °C							Aw	Aw+Rm	Aw+Rg	Aw+Ck	Aw+Cz	Aw+Mix	Aw+St
	20 °C							Pn	Aw+Rm	Aw+Rg	Aw+Ck	Aw+Cz	Aw+Mix	Aw+St

(**Rm=** *Rhodotorula mucilaginosa;* **Rg=** *Rhodotorula glutinis;* **Ck=** *Candida krusei;* **Cz=** *Candida zeylanoides;* **Mix**= Rm+Rg+Ck+Cz; **St**=starter; **Pn**= *Penicillium nordicum;* **Aw=** *Aspergillus westerdijkiae;* **1%=** 1% NaCl; **3%=** 3% NaCl)

3.4.2 Influence of the matrix

After analysing the results obtained in the preliminary assay, 15 ºC with 1 % NaCl and co-inoculation of fungus with *C. krusei* and *R. glutinis* were found to be the less interesting for further analysis. For that reason, the commercial starter, *R. mucilaginosa*, *C. zeylanoides* and a mix of both yeasts were selected to test the influence of the matrix on fungal growth and OTA production. For this purpose, three different matrices were used: Ind, Trad and Ham all with 3 % salt added (see **table 3.1** for media characteristics). Ham was introduced in the study as a positive control, because this matrix had been previously used by our research team to determine ecophysiological conditions of *P. nordicum* and *A. westerdijkiae*, in terms of growth and OTA production (Vipotnik et al, submitted). Trad was used as matrix similar to Ind -. Co-inoculations were prepared as described in **section 3.2.1**, and incubated at 20 °C for 14 days (**table 3.3**).

Table 3.3 Different conditions of co-inoculation

	Ham	Ind	Trad
Control	Pn	Pn	Pn
	Pn+ Mix	Pn+ Mix	Pn+ Mix
Test	Pn+ Cz	Pn+ Cz	Pn+ Cz
	Pn+ Rm	Pn+ Rm	Pn+ Rm
	Pn+ St	Pn+ St	Pn+ St
Control	Aw	Aw	Aw
	Aw+ Mix	Aw+ Mix	Aw+ Mix
Test	Aw+ Cz	Aw+ Cz	Aw+ Cz
	Aw+ Rm	Aw+ Rm	Aw+ Rm
	Aw+ St	Aw+ St	Aw+ St

(Rm = *R. mucilaginosa;* **Cz =** *C. zeylanoides;* **Mix** = Rm+Cz; **St =** starter; **Pn =** *P. nordicum;* **Aw =** *A. westerdijkiae*)

3.4.3 Mechanisms of action

To understand the mechanisms of action of the test microorganisms, fungi were co-inoculated with each test organism in different ways: i) Effect of incorporated live cells was tested by incorporation of living cells in the medium, as positive control (the same condition as previously tested); ii) Effect of incorporated dead cells, to determine if the effect on fungi was caused by a structural compound of the cell; iii) Effect of cell-free culture filtrate, by using as culture medium the filtered broth where yeast/starter were previously grown, to determine if the effect was caused by a compound previously produced by the yeast/starter and diffused to the medium; iv) Effect of diffusible compounds, fungi and yeast/starter where inoculated at a given distance, to study if the effect was caused by yeast/starter compounds being produced simultaneously with the fungus and being diffused towards the fungus; and v) Effect of volatile compounds, fungi and yeast/starter were inoculated without direct contact (cut culture medium), to verify if the microorganisms produced a volatile compound which influenced fungal growth and OTA production.

Because this assay was used to establish a proof-of-concept, only two culture media (ham and Trad with 3 % NaCl) and one temperature (20 ºC) were used with *C. zeylanoides* and starter culture. The selection of these conditions was due to the inability of *P. nordicum* to produce OTA in Ind medium and the difficulty of *R. mucilaginosa* to grow in ham medium. For these assays, each fungus was inoculated in 9 cm Petri dishes by two-point inoculation with 2 µL spore suspension, in triplicate. After co-inoculation all petri dishes were incubated at 20 °C for

14 days. A general overview of procedures for each of the tests is given below, and is summarised in **Figure 3.3.**

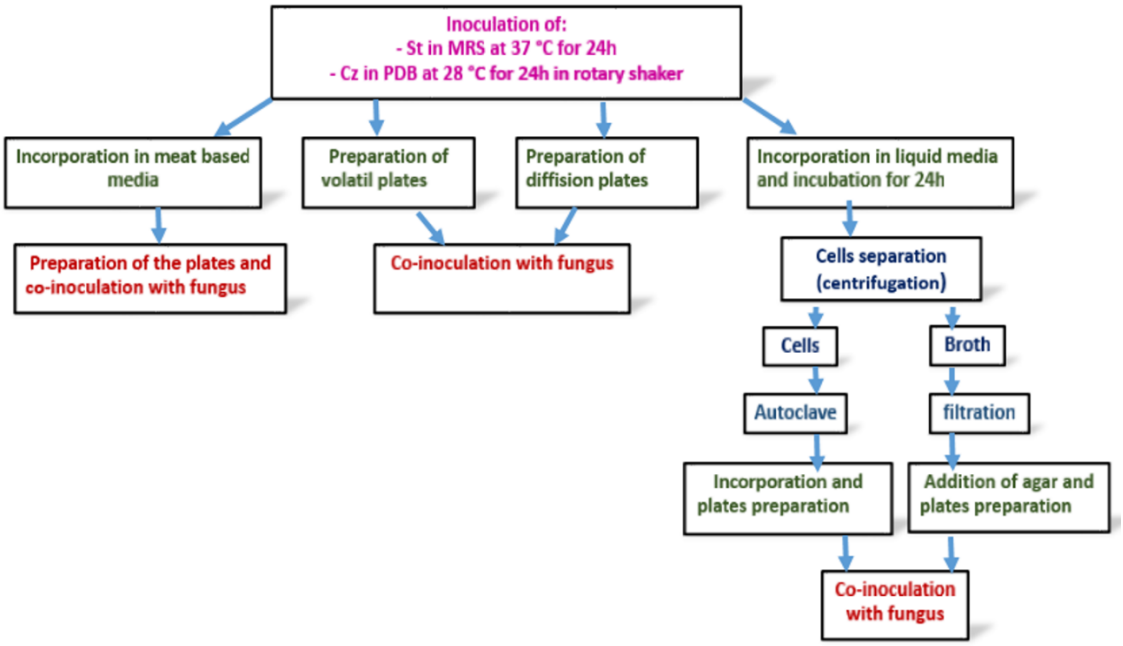


Figure 3.3: Summary of the different methods of co-inoculation for the determination of mechanism of action of starter and *C. zeylanoides* against *P. nordicum* and *A. westerdijkiae*

3.4.3.1 Effect of incorporated live cells

C. zeylanoides and starter culture cell suspensions were inoculated into each medium **(figure 3.4)**. The inocula were prepared as described in **section 3.2**. Fungi were inoculated as previously described, except that only with two opposite spots of 2 µL of spore suspension.

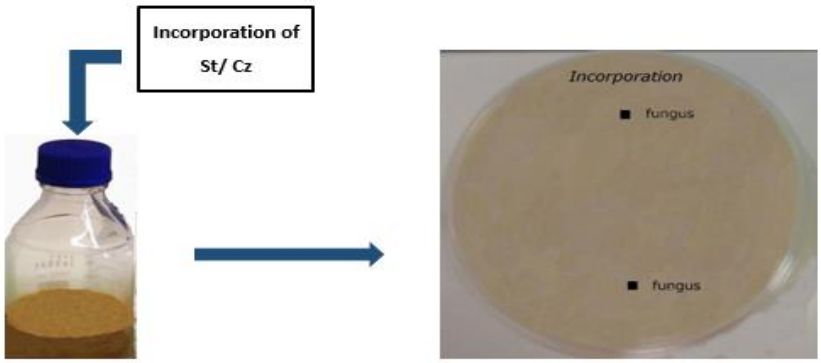


Figure 3.4 Incorporation of live cells and co-inoculation with fungus

3.4.3.2 Effect of incorporated dead cells

C. zeylanoides and starter culture were inoculated in 150 mL of Ham and Trad broth, and incubated at 28 °C in a rotary shaker (120 rpm) and at 37 °C, respectively. After 24 h of incubation, cells were centrifuged at 5000 rpm for 10 min (**figure 3.5**). The supernatant (broth) was transferred into a sterile bottle under aseptic conditions (to be further used; see **section 3.4.3.3**). The resulting pellet was resuspended in 10 mL of sterile water and autoclaved at 120 °C for 15 minutes to kill the cells. Dead cells were incorporated in 150 mL of Ham and Trad solid media and co-inoculated with each fungus **(figure 3.6).**

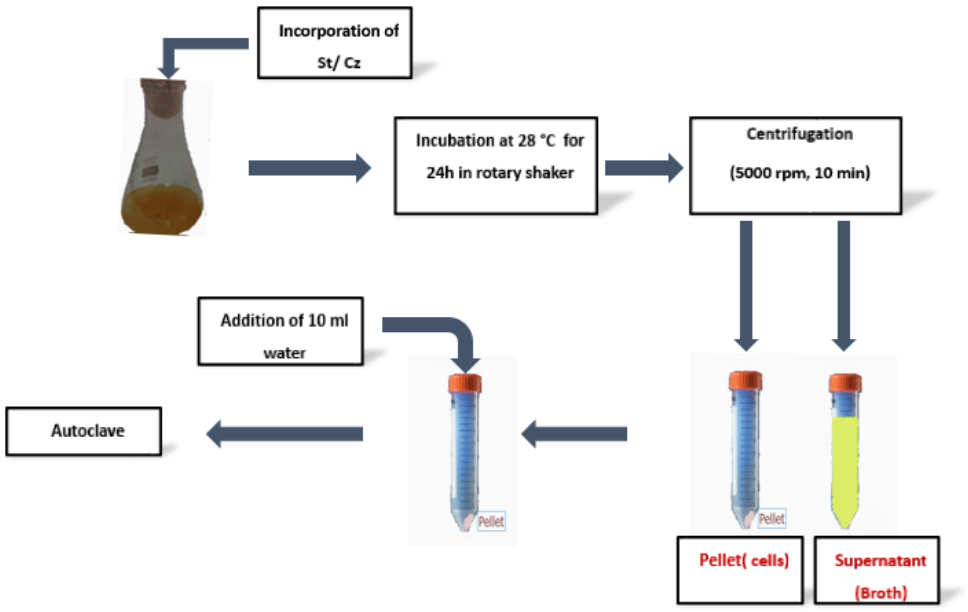


Figure 3.5 Cell separation from the broth

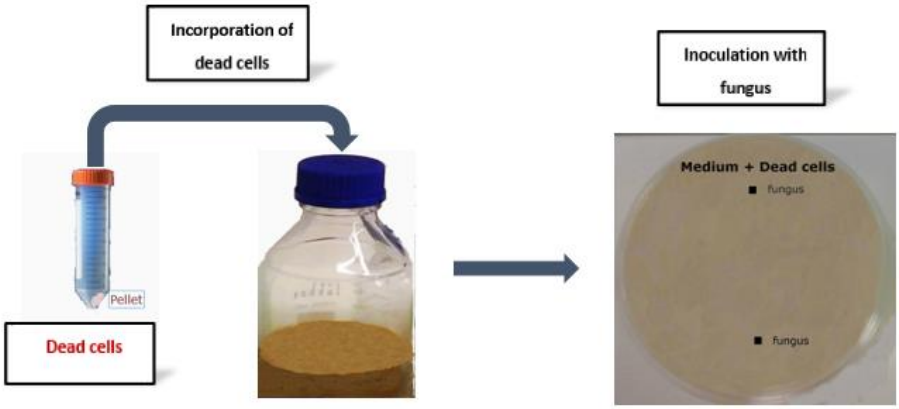


Figure 3.6 Incorporation of dead cells and co-inoculation with fungus

3.4.3.3 Effect of cell-free broth

The cell-free broth obtained in **section 3.4.3.2** was used in this section. To ensure the total elimination of cells, the broth resulting from cell centrifugation was filtered with 0.22 µm filters. Two percent of dissolved agar was added to the filtered broth and poured in 9 cm Petri dishes. Fungi were inoculated as in **section 3.4.3.2 (figure 3.7)**.

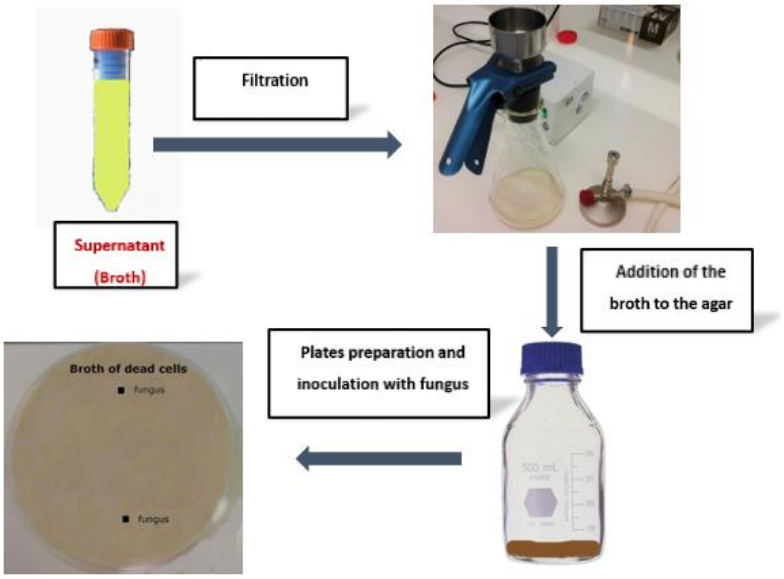


Figure 3.7 Filtration of the Broth and co-inoculation of fungus

3.4.3.4 Effect of diffusible compounds

To verify if yeast or starter influenced fungal growth and OTA production by producing diffusible compounds, 2 µL of spore suspension and 2 µL of starter or *C. zeylanoides* were inoculated in opposite quadrants of the Petri dish as shown in **figure 3.8**.

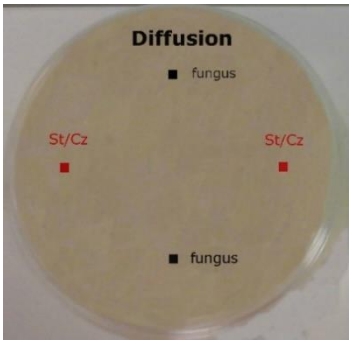


Figure 3.8 Co-inoculation of microorganisms for detection of diffusible compounds with effect on fungi

3.4.3.5 Effect of volatile compounds

Twenty mL of each meat-based medium were plated in 9 cm petri dishes and then divided into 4 quadrants by cutting 3 mm lanes of agar perpendicularly, to avoid the contact of diffusible compounds potentially produced by test microorganisms with the fungus. Then, 2 µL of spore suspension were added in two opposite quarters and 2 µL of yeast or starter suspension were added to the other 2 opposite parts **(figure 3.9).**

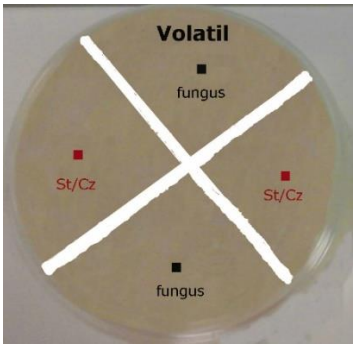


Figure 3.9 Co-inoculation of microorganisms for detection of volatile compounds with effect on fungi

3.5 OTA analysis

3.5.1 OTA extraction

OTA was extracted from plates after 14 days of incubation. Three agar plugs were removed from the inner, middle and outer areas of the colony, weighted and extracted with 1.5 mL of methanol as described by Bragulat et al. (2001). The agar plugs were maintained in methanol for one hour and vortexed every 15 min. The extracts were filtered by PTFE 0.2 µm syringe filters and stored at 4 ºC until further analysis (**Figure 3.10**).

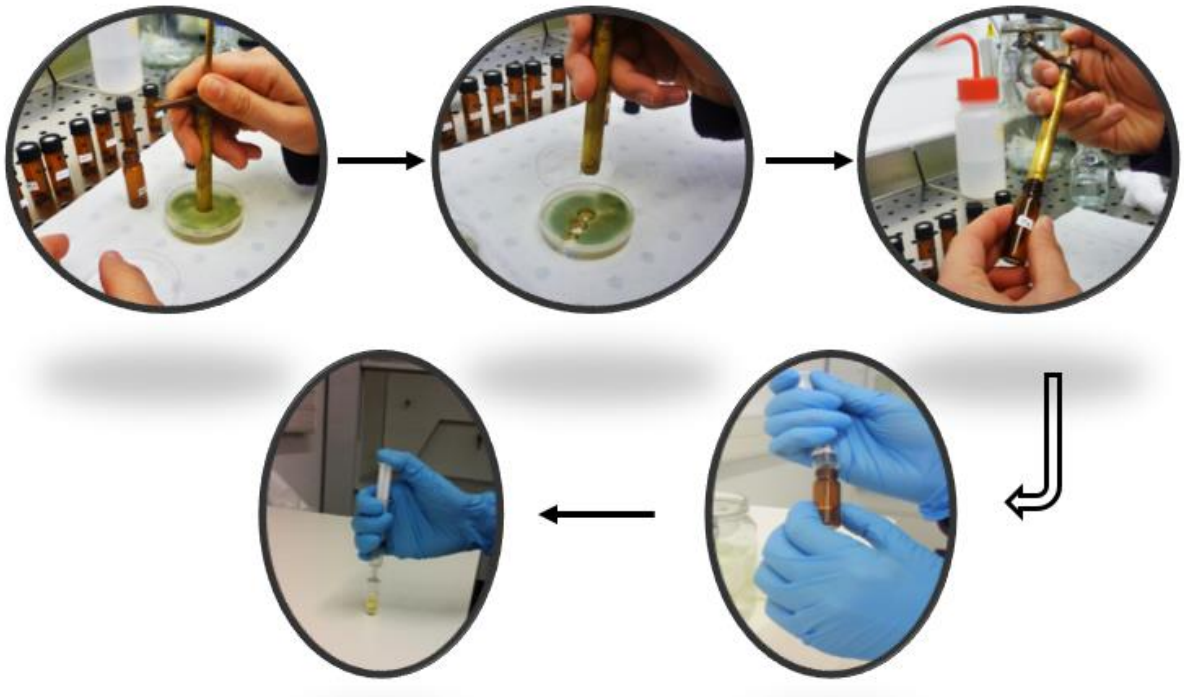


Figure 3.10 OTA extraction steps

3.5.2 OTA detection and quantification by High Performance Liquid Chromatography (HPLC)

OTA was analysed by High Performance Liquid Chromatography (HPLC) system equiped with: Smartline pump (1000, Knauer, Berlin, Germany), degasser system (Smartline manager 5000), auto-sampler (AS-2057, Jasco, Easton, MD, USA), and a fluorescence detector (FP-2020, Jasco) set to λex 330 nm and λem 463 nm. Data were analysed using Clarity 2.4 Software (DataApex, Prague, Czech Republic). The chromatographic separation was achieved using an isocratic elution with a C18 reverse-phase column (100 x 4.6 mm, Merck Chromolith Performance, Darmstadt, Germany) operating at 35 °C (7971 R Grace oven). The mobile phase consisted of an isocratic programme of water: acetonitrile: acetic acid (29.5:70:0.5, v/v/v) and was pumped at 0.8 mL/min for a total run time of 4 minutes. The injection volume was 10 µL. Under these conditions, retention time for OTA was 2.2 minutes.

Linearity, limit of detection (LOD) and limit of quantification (LOQ) were determined by three series of analyses, using 11 standard solutions with concentrations ranging from 0.05 ng/mL to 100 ng/mL. The calibration curve was $y = 820002x + 444102$ and $R^2 = 0.9979$. LOD and LOQ were calculated according to the following equations (Taverniers *et* al, 2004):

LOD = 3 x (sa/b) and LOQ = 10 x (sa/b), where sa is the standard deviation of the intercept of the regression line obtained from the calibration curve, and *b* is the slope of the line. LOD and LOQ were calculated as 0.5 and 1.5 ng/mL, respectively. OTA was quantified by gram of agar, taking into consideration the weight of the agar plugs used for extraction.

3.6 Statistical analysis

Statistical analysis was performed using IBM® SPSS® Statistics v.22.0 software (Armonk, NY: IBM Corp.). For the comparison of means, samples were first tested for normal distribution by Shapiro-Wilk test ($n < 30$) and for homogeneity of variances by Levene's test. Since samples generally followed these criteria, t-student test and One-way ANOVA were used for comparison of means for 2-level variables and for more than 2-level variables, respectively. Two- way ANOVA was used to test interaction between two factors Post-hoc analyses were performed with Dunett test (to create confidence intervals for differences between the mean of each factor level and the mean of a control group). In all cases, statistical significance was established at $p \leq 0.05$. Two-way interactions between factors appear as an antagonistic (negative coefficient) or a synergistic effect (positive coefficient).

4. RESULTS AND DISCUSSION

4.1 Yeast identification

One hundred yeasts previously isolated from traditional sausage were morphologically characterised and grouped in terms of colony and cell morphology. From the groups having the highest number of isolates, ten yeasts were selected for biochemical identification by the API 20 C AUX system. The following ten yeasts were identified: *Candida albicans*, *Candida colliculosa*, *Candida krusei* (2 isolates), *Candida zeylanoides* (2 isolates), *Cryptococcus laurentii*, *Rhodotorula glutinis*, and *Rhodotorula mucilaginosa* (2 isolates). Although the API 20 C system is widely used in yeast identification, its scope is limited to the clinical field. So, biochemical identification was compared with morphological characterisation to confirm agreement between both methods of identification.

The morphological and biochemical methods are still useful technics for yeast identification, however many food yeast species cannot be reliably distinguished on the basis of phynotypic features only, and it is sometimes required to confirm the biochemical identification by other methods like molecular identification. Progress in molecular biology has provided a large number of DNA based techniques for identifying and characterising yeasts. These include DNA–DNA hybridisation, electrophoretic karyotyping, RFLPs of chromosomal DNA and, most frequently, genomic, mitochondrial and ribosomal DNA sequencing. Within these molecular techniques the sequencing of the D1/D2 domain of 26S rRNA gene has been used as universal DNA target to identify yeast isolates to the genera or species level (Kurtzman and Robnett, 1998; Leaw et al, 2006).

Based on preliminary phenotypic identification, four yeasts – *C. zeylanoides*, *C. krusei*, *R. glutinis* and *R. mucilaginosa* – were selected for further studies based on non-pathogenicity and adaptability to the matrix. The identification of the four yeasts was further confirmed by molecular analysis by sequencing the D1/D2 domain of 26S rRNA gene. As shown in **Figure 4.1**, intended DNA amplicons of all strains were successfully amplified with primers NL-1 and NL-4 (Kurtzman and Robnett, 1998). PCR products were found to have the expected size of *ca.* 550-600 bp.

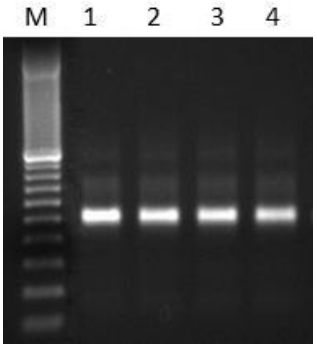


Figure 4.1 Visualization of D1/D2 amplicons amplified with primers NL-1 and NL-4. **M:** Molecular size marker (Bioron); **1:** *R. mucilaginosa*; **2:** *R. glutinis*; **3:** *C. krusei*; **4:** *C. zeylanoides*

Because no method is flawless, correct identification of microbial isolates strongly relies on an integrative approach of several identification methods. For the yeast isolate biochemically identified as *C. zeylanoides* the morphological, biochemical and genetic analyses were in agreement, as total similarity (100%) was observed with *C. zeylanoides* sequences in NCBI database. The accuracy of identification of *C. zeylanoides* isolated from dry fermented sausage was thus confirmed.

On the other hand, some discrepancies were found between molecular and biochemical identification for the other three isolates. The API 20 C AUX system identified *Rhodotorula* yeasts as *R. glutinis* and *R. mucilaginosa*, with high levels of confidence (more than 86 %). However, BLAST searches of the D1/D2 sequences showed 100 % similarity of both species with the *Rhodotorula graminis*/*babjevae/glutinis* complex. This high similarity level could be because they are phylogenetically very close species, and in this case the D1/D2 region was not sufficiently discriminatory. This problem has been previously reported by other authors (e.g. Daniel and Meyer 2003; Leaw et al, 2006). In fact, it is not unusual to find that genetic barcode sequences (i.e. sequences universally accepted as species "fingerprints") are not sufficiently discriminatory between genetically similar species (Rodrigues et al, 2011). For that reason, macro and micromorphology of the studied yeasts was used to confirm *R. glutinis* and *R. mucilaginosa* as the final identification.

The yeast biochemically identified as *C. krusei* was molecularly identified as *C. zeylenoides*. In spite of this, the morphological characteristics showed a significant difference between the subject yeast and *C. zeylanoides* and remarkable similarity with the

common properties of *C. krusei*. It is logical to conclude that the yeast isolates in this work are *C. krusei*, *C. zeylanoides*, *R. glutinis* and *R. mucilagonosa*.

Results obtained for the various methods of identification and the final identification achieved by an integrative approach are shown in **table 4.1**.

Table 4.1 Morphological, biochemical and molecular identification of the four yeasts used in the study

Morphology		**API 20 C AUX**	**D1/D2**	**Integrated ID**
Macromorphology	**Micromorphology**	**ID (% identity)**	**ID (% identity)**	
Colony red to orange, mucose	Cells oval, polar budding	*Rhodotorula glutinis* (86.3%)	*Rhodotorula graminis/babjevae/ glutinis* (100%)	*R. glutinis*
Colony pink to red, mucose, smooth, glistening	Cells oval, polar budding	*Rhodotorula mucilaginosa* (96.1%)	*Rhodotorula graminis/babjevae/ glutinis* (100%)	*R. mucilaginosa*
Colony white to cream, dry	Cells ellipsoidal, multiple budding	*Candida krusei* (98.9%)	*C. zeylanoides* (100%)	*C. krusei*
Colony white to cream, dry	Cells oval, multiple budding	*Candida zeylanoides* (99.9%)	*C. zeylanoides* (100%)	*C. zeylanoides*

4.2 Effect of microorganisms on fungal growth and OTA production

4.2.1 Preliminary co-inoculation assays

This study aimed to determine the effect of a selected group of microorganisms on growth and OTA production by *P. nordicum* and *A. westerdijkiae*. For that purpose, the four selected sausage-native yeasts, a mix of those yeasts and a starter culture commercially used in sausage fermentation and curing process were co-inoculated with each of the fungus under two different temperatures (15 and 20 ºC) and two salt concentrations (1 and 3 %).

The results of fungal growth (in terms of colony diameter) in Ind media with 1 % and 3 % NaCl incubated at 15 and 20 ºC are represented in **figure 4.2**.

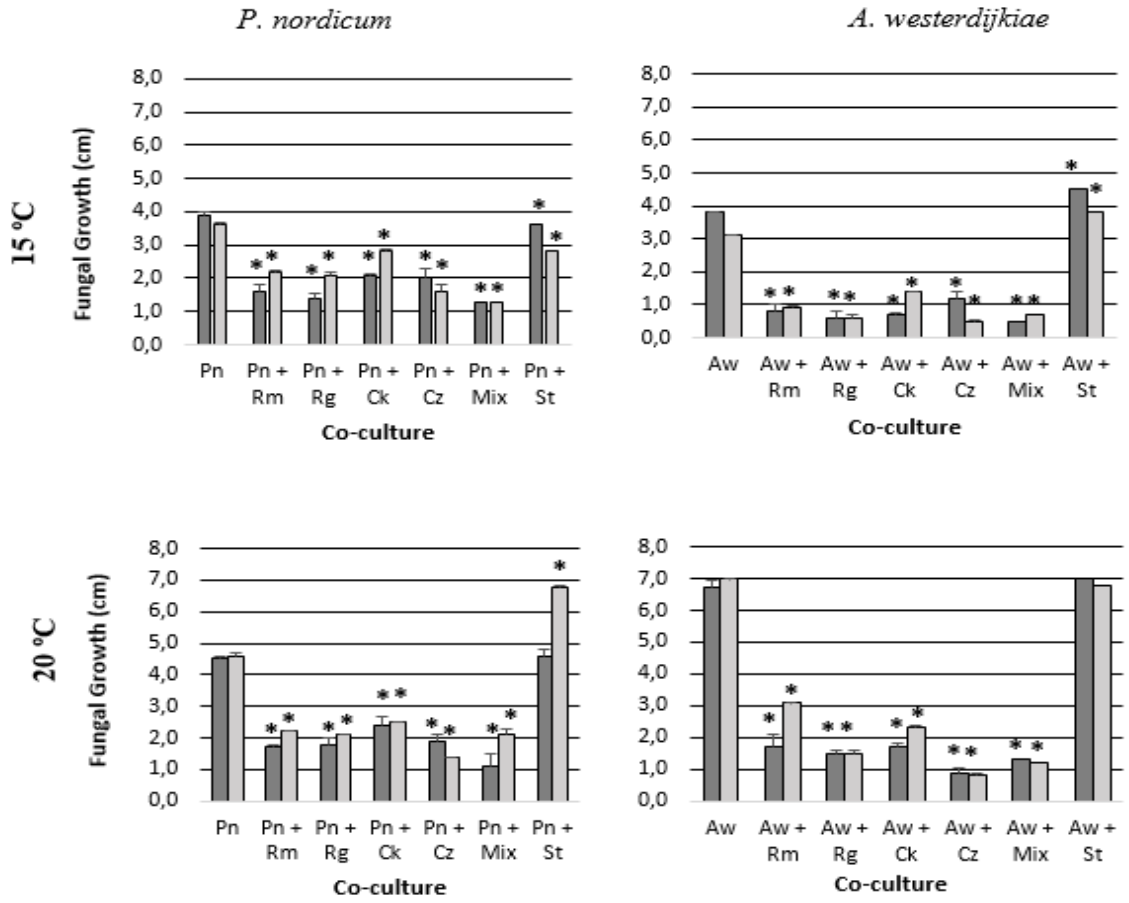


Figure 4.2 Growth of *P. nordicum* (left) and *A. westerdijkiae* (right) in Industrial sausage-based medium supplemented with 1 % NaCl (dark bars) and 3 % NaCl (light bars), incubated at 15 ºC (top) and 20 ºC (bottom), after 12 days of incubation. In all cases, results are the average of six replicates; standard deviation is indicated as vertical thin lines
* Significantly different from control (fungus only), $p \leq 0.05$

After 12 days of incubation, a significant inhibition effect was detected by all yeasts and Mix on *P. nordicum* and *A. westerdijkiae* growth under all tested conditions. From these results the inhibiting effect of yeasts on fungal growth could be confirmed. This reduction in fungal growth could be due to competition for nutrients and space (Hernández-Montiel et al, 2010), production of hydrolytic enzymes, killer toxins (Masih and Paul, 2002) or secretion of volatile compounds (Masoud et al, 2005; Taczman-Brückner et al, 2005; Fialho et al, 2009). In previous researches, several yeasts such as *Hyphopichia burtonii* and *C. zeylanoides* were reported also as strong inhibitors of *P. nordicum* growth in dry-cured ham and dry fermented sausage (Virgili et al, 2012; Andrade et al, 2014; Núñez et al, 2015).

Starter culture had a variable effect under the different conditions. Tested *P. nordicum* was inhibited under all conditions except at 20 ºC and 3 % NaCl, where growth was significantly

stimulated. On the other hand, starter significantly stimulated growth of *A. westerdijkiae* at 15 ºC (1 and 3% NaCl), but not at 20 ºC. The commercial starter culture, which is frequently used in fermentation process of meat products, is composed by *Pediococcus pentosaceus, Lactobacillus sakei, Staphylococcus carnosus, Staphylococcus xylosus* and *Debaryomyces hansenii*. Species of genera *Pediococcus* and *Lactobacillus* have been studied as potential biocontrol agents against fungi and mycotoxins in several food matrices, with inhibiting effects being observed (Abrunhosa et al, 2014; Ngang et al, 2015; Pereira et al, 2016). *D. hansenii* has also previously shown ability for inhibition of *P. nordicum* growth (Virgili et al, 2012; Andrade et al, 2014; Simoncini et al, 2014).

The mechanism of action of starter culture is not well defined at this stage of this work and to understand this unexpected stimulation, the pH of the medium was determined giving pH=4.4. The starter culture for fermented sausage must be maintained at range of pH between 4.8 and 5 to ensure that microorganisms maintain their activity over a long time (Starter Cultures for Making Fermented Sausages, URL: http://www.meatsandsausages.com/sausage-types/fermented-sausage/cultures, accessed 25-06-2016). The mechanism behind this inhibition could be that it produces a Killer toxin affecting the fungus (Masih and Paul, 2002). However, Marquina et al (2001) proved that 25% of the killer activity were lost after just 5 days of incubation at 20°C with pH 4, and Turk et al (2007) reported that low pH (pH 4) resulted in a significant decrease in membrane fluidity, and changes in the lipid composition: increase in ergosterol content and sterol-to-phospholipid ratio, together with the decrease in anionic phospholipids concluding that at low pH cells are struggling to survive. For that, starter culture maybe loses its effectiveness. The stimulation of *P. nordicum* growth may be due to the presence of more nutrients than in control medium provided by inactive or dead starter culture cells.

The results of OTA production by *A. westerdijkiae* after 14 days of incubation in Ind media with 1 % and 3 % NaCl at 15 and 20 ºC are represented in **figure 4.3**. No detectable OTA was produced by *P. nordicum* under these test conditions. **Figure 4.4** shows examples of OTA chromatograms for OTA standard (50 ng/mL), and OTA positive (Aw+Rm / Ind / 3% NaCl / 15 ºC) and negative (Aw / Ind / 3 % NaCl / 15 ºC) samples.

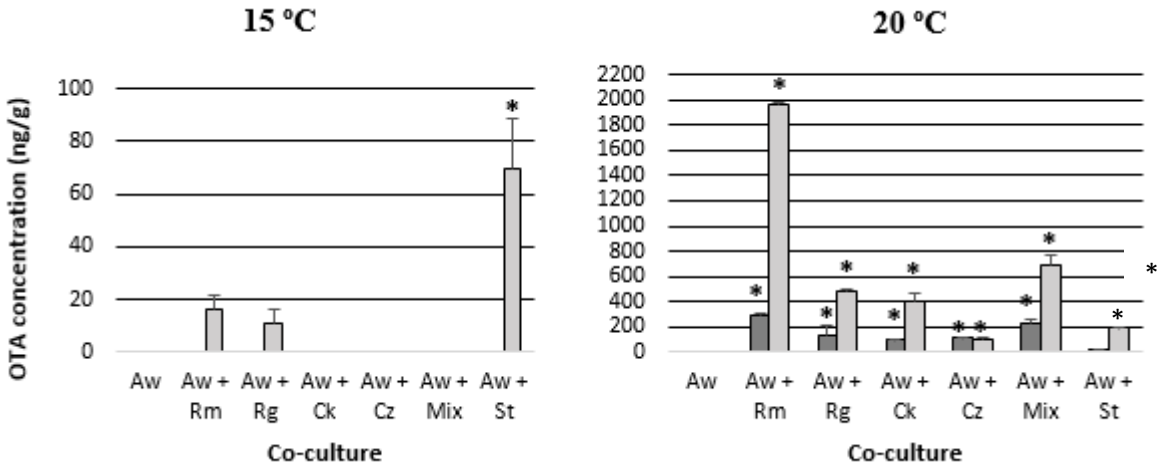


Figure 4.3 Ochratoxin A production by *A. westerdijkiae* in Industrial sausage-based medium supplemented with 1 % NaCl (dark bars) and 3 % NaCl (light bars) at 15 ºC (left) and 20 ºC (right), after 14 days of incubation. In all cases, results are the average of six replicates; standard deviation is indicated as vertical thin lines

* Significantly different from control (fungus only), $p \leq 0.05$

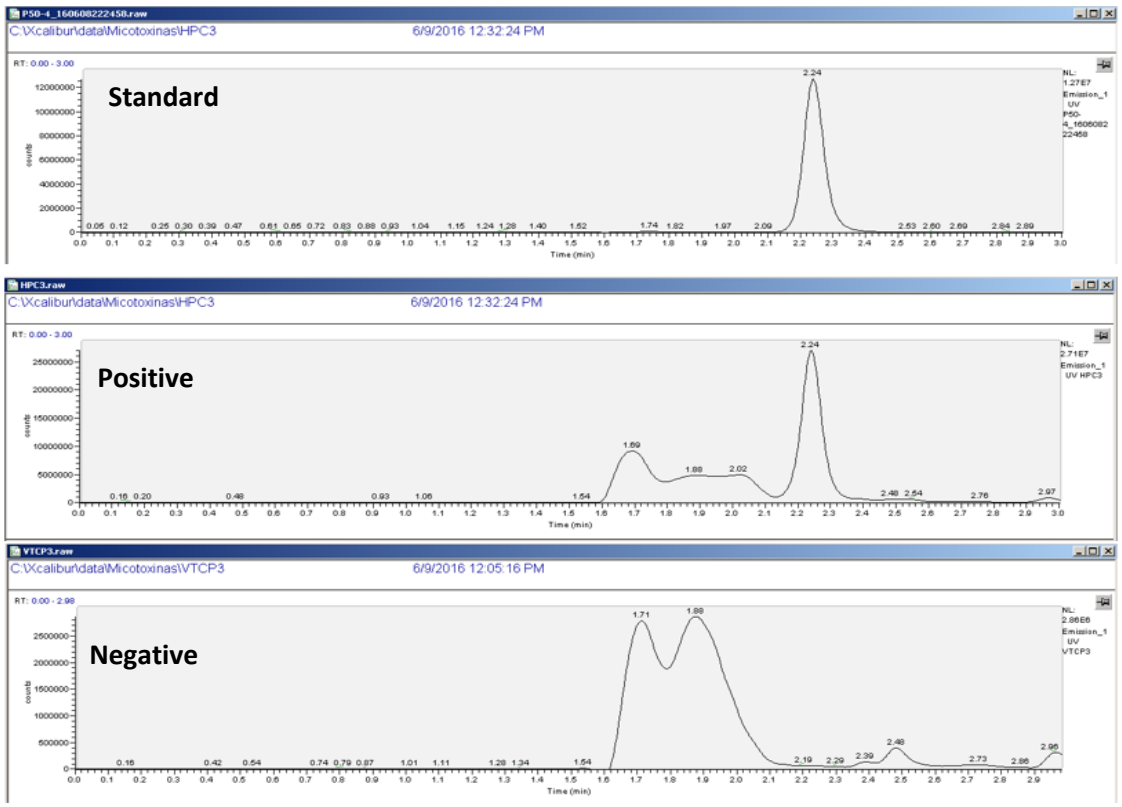


Figure 4.4 OTA chromatogram for standard (50 ng/mL) (top), positive sample (Aw+Rm / Ind / 3% NaCl / 15 ºC) (middle) and negative sample (Aw / Ind / 3 % NaCl / 15 ºC) (bottom). OTA is represented by the peak at retention time 2.24 minutes

Unexpectedly, the pure (axenic) culture of *A. westerdijkiae* and *P. nordicum* were unable to produce detectable amounts of OTA in Ind medium, under any of the tested conditions. Even though *A. westerdijkiae* is usually considered a strong contaminant of carbon-rich plant-based products (Gil-Serna et al, 2015), it can also contaminate dry-cured hams (Scaramuzza et al, 2015; P. Rodrigues, pers. Commun.). Furthermore, studies developed by our research team demonstrated that both fungi were able to grow and produce high amounts of OTA in dry-cured ham-based medium under a wide range of conditions (Vipotnik et al, submitted). The inability of fungi to produce OTA in industrial sausage-based medium is probably due to the composition of this meat product, either by the effect of natural composition (lipid and protein contents) or by the effect of added compounds (preservatives, condiments).

At 15 ºC, detectable amounts of OTA were produced by *A. westerdijkiae* only at 3 % NaCl and the highest concentration was recorded in the co-culture with the starter (70 ng/g). On the other hand, when incubated at 20 ºC, OTA production by this fungus was significantly stimulated by all yeasts and Mix, with *R. mucilaginosa* being the most inducing agent (1959 ng/g). The observed OTA enhancement effect of yeasts and starter was also unexpected, mostly because those microorganisms have been tested and proved as potential biocontrol for other OTA-producing species. Even though those microorganisms have inhibited *A. westerdijkiae* growth, it cannot be assumed that OTA would also be inhibited (**figures 4.2 and 4.3**). In fact, in a study of *A. westerdijkiae* ecophysiology in plant-derived products (coffee, grapes, paprika, barley, maize and anise), Gil-Serna et al (2015) found no correlation between fungal growth and mycotoxin production. Growth inhibition can be considered a stress factor to the fungus, which potentially results in the activation of the secondary metabolism as a stress response, with consequent OTA production.

P. nordicum was also unable to produce OTA under any condition but, contrary to *A. westerdijkiae*, no stimulation of OTA was observed in any of the co-cultures. This was also unexpected, since this strain was isolated from Spanish ham, and has been reported as OTA producer in meat-based media (A. Rodríguez, pers. commun.; Vipotnik et al., submitted). The same effects of sausage composition previously described as responsible for OTA inhibition in *A. westerdijkiae* are probably acting on *P. nordicum* also.

The results of statistical analyses of the influence of two temperatures (15 and 20 ºC) and a_w (salt concentration) 0.98 (1 %) and 0.95 (3 %) on fungal growth and OTA production are represented in **table 4.2**.

Table 4.2 Statistical analyses of the influence of temperature, water activity and the interaction of both on *P. nordicum* growth and *A. westerdijkiae* growth and OTA production ($p < 0.05$)

Species	Test variable	Factor	Statistical set	*p*-value
Pn	**Growth**	Temperature	One way ANOVA	0.01
		Aw	One way ANOVA	0.122
		Temperature*aw	Two way ANOVA	0.277
Aw	**Growth**	Temperature	One way ANOVA	0.000
		Aw	One way ANOVA	0.923
		Temperature*aw	Two way ANOVA	0.455
	OTA	Temperature	One way ANOVA	0.000
		Aw	One way ANOVA	0.01
		Temperature*aw	Two way ANOVA	0.004

Interactions between available water and temperature are fundamental because they represent the two-dimensional niche in which fungi may be able to germinate, grow and actively compete for the allocation of the available resources (Samapundo et al, 2007). It is also generally well agreed that, in contrast to bacterial growth, a_w is the most significant factor controlling fungal growth (Samapundo et al., 2007). Despite the fact that a_w is usually considered the most influential factor on fungal growth, in the present study a significant influence of temperature on growth of both fungi was found, but no significant effect of a_w or interaction "temperature*a_w" was detected. This could be explained by the low range of a_w used (0.95 and 0.98) which are both good conditions for *P. nordicum* and *A. westerdijkiae* growth. However, the significant difference between 15 and 20 ºC depends on the optimum condition for *A. westerdijkiae* and *P. nordicum*. In fact, *A. westerdijkiae* is more adapted to temperate climates, while *P. nordicum* is associated with colder climates, and for that reason *A. westerdijkiae* showed significantly more growth at 20 ºC (7 cm) than at 15 ºC (3.9 cm), while *P. nordicum* showed no growth limitations for both temperatures.

A significant influence of temperature, a_w and interaction "temperature*a_w" was found for OTA production by *A. westerdijkiae*, being 3 % NaCl at 20 ºC the optimum condition. The significant influence of a_w/salt is expected, since OTA biosynthesis is naturally activated under sub-optimal, weak stress conditions (3 % salt) (Schmidt-Heydt et al, 2007).

4.2.2 Influence of the matrix

Based on the unexpected results obtained and described in **section 4.2.1** it was found necessary to run further experiments to confirm the inability of both fungi to produce OTA in sausage-based medium, and the effect of matrix on OTA induction by test microorganisms. For that, different matrices – industrial sausage (the same as the previously used), traditional sausage and ham – were tested.

The results of *P. nordicum* and *A. westerdijkiae* growth in Ham-, Industrial sausage- and Traditional sausage-based media (further mentioned as Ham, Ind and Trad respectively) with 3 % NaCl incubated at 20 ºC are represented in **figures 4.5**, **4.6** and **4.7**. It must be remarked that, for this particular study, *R. mucilaginosa* showed limited growth in Ham, either alone or in the Mix (Cz+Rm), for both *P. nordicum* and *A. westerdijkiae* co-cultures. Based on this fact, results obtained for these conditions were not subject of analysis.

Yeasts and starter significantly inhibited *P. nordicum* growth in Ham, Ind and Trad media with the exception of starter in Trad. On the other hand, *A. westerdijkiae* growth was significantly stimulated by the presence of starter, whereas significant decrease was exercised by yeasts in all media.

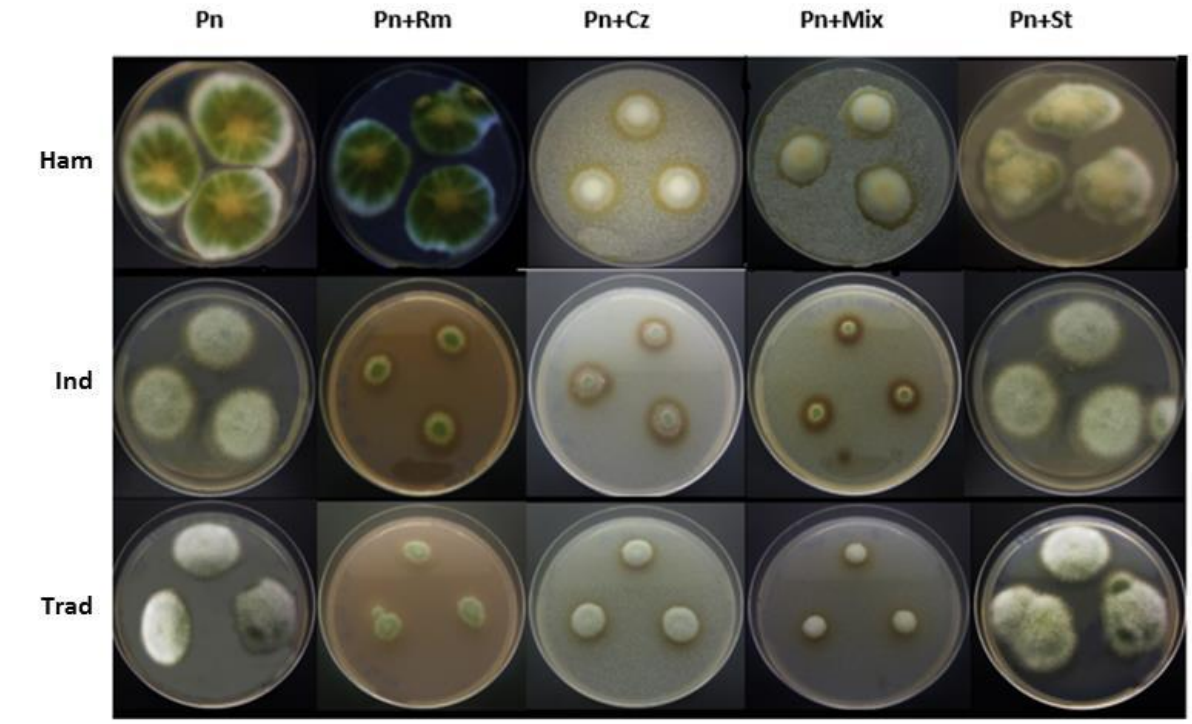


Figure 4.5 Growth of *P. nordicum* under different conditions in three different media – Ham (top), Industrial (middle) and Traditional (bottom) – at 20 ºC and 3% NaCl, after 14 days of incubation

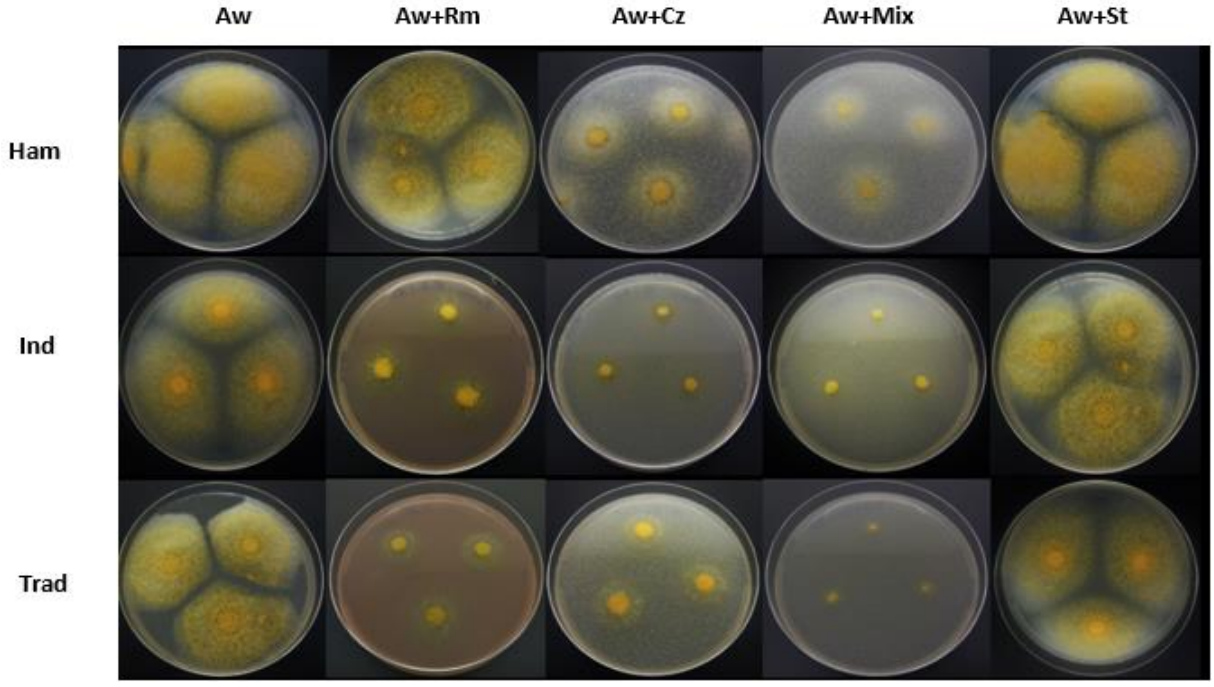


Figure 4.6 Growth of *A. westerdijkiae* under different conditions in three different media – Ham (top), Industrial (middle) and Traditional (bottom) – at 20 ºC and 3% NaCl, after 14 days of incubation

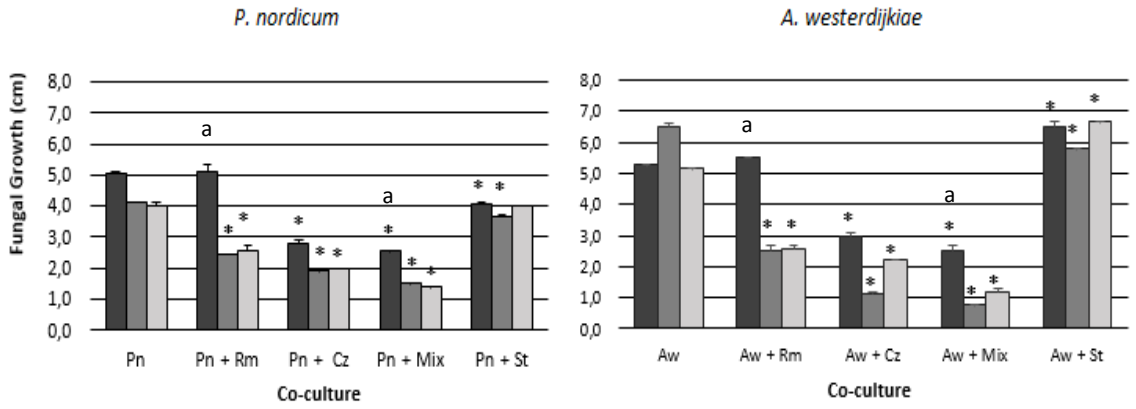


Figure 4.7 Growth of *P. nordicum* (left) and *A. westerdijkiae* (right) in different media: Ham (black bars), Industrial (grey bars) and Traditional (light bars), at 20 ºC and 3% NaCl, after 14 days of incubation. In all cases, results are the average of six replicates; standard deviation is indicated as vertical thin lines
* Significantly different from control (fungus only), $p \leq 0.05$
[a] no growth of Rm

Results of OTA production by *P. nordicum* and *A. westerdijkiae* in the three media with 3 % NaCl incubated at 20 ºC are represented in **figures 4.8**.

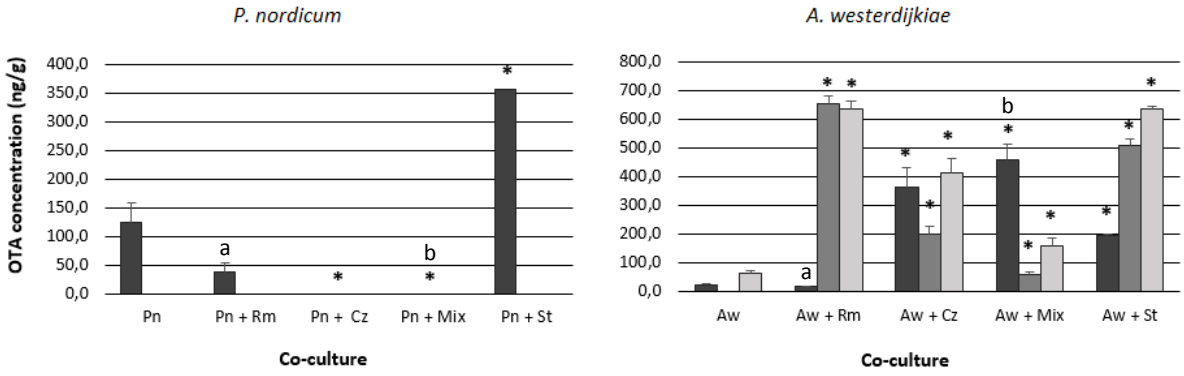


Figure 4.8 Ochratoxin A production by *P. nordicum* (left) and *A. westerdijkiae* (right) in different media: Ham (black bars), Industrial (grey bars) and Traditional (light bars), at 20 ºC and 3% NaCl. In all cases, results are the average of six replicates; standard deviation is indicated as vertical thin lines
* Significantly different from control (fungus only), $p \leq 0.05$
[a] no growth of Rm
[b] no growth of Rm; effect probably limited to Cz

As reported for the previous test, *P. nordicum* was not able to produce detectable amounts of OTA in Ind, and the same happened in Trad media, for all conditions tested. However, when in pure culture, it produced relatively high amounts of the toxin in Ham medium (123 ng/g). In this matrix, *C. zeylanoides* significantly inhibited OTA production by *P. nordicum*, while the starter culture strongy stimulated its production. *C. zeylanoides* had been previously reported

to have strong inhibition effect on *P. nordicum* growth and OTA production on ham-based media (Andrade et al, 2014; Simoncini et al, 2014) and on other synthetic salt-rich media (Virgili et al, 2012; Núñez et al, 2015). These studies have all been done with single biocontrol agents and, to our knowledge, no studies have determined the effect of mixed agents, as is the case of the starter culture (which is a mix of microorganisms composed of yeasts and bacteria) used in this study. For *P. nordicum*, it seems like the starter, although reducing growth - or probably because of it, as previously mentioned -, stimulates fungal secondary metabolism and consequent OTA production.

A different behaviour was found for *R. mucilaginosa*, *C. zeylanoides* and starter co-inoculated with *A. westerdijkiae*. While yeasts significantly reduced fungal growth, OTA production was highly and significantly stimulated in all culture media. *R. mucilaginosa*, even though generally showing less effect on growth inhibition than *C. zeylanoides*, exerted a stronger effect on OTA stimulation. Starter culture also stimulated OTA production, with a stronger effect on both sausage-based media than on Ham.

The statistical analysis of the influence of three different matrices on fungal growth and OTA production are presented in **table 4.3**.

Table 4.3 Statistical analyses of the influence of matrix on *P. nordicum* and *A. westerdijkiae* growth and OTA production ($p < 0.05$)

Species	Test variable	Factor	Statistical set	*p*-value
Pn	**Growth (cm)**	Ham-Ind	One way ANOVA	0
		Ham-Trad	One way ANOVA	0
		Ind-Trad	One way ANOVA	0,982
	OTA	Ham-Ind	One way ANOVA	0,002
		Ham-Trad	One way ANOVA	0,002
		Ind-Trad	One way ANOVA	1
Aw	**Growth (cm)**	Ham-Ind	One way ANOVA	0,053
		Ham-Trad	One way ANOVA	0,154
		Ind-Trad	One way ANOVA	0,877
	OTA	Ham- Ind	One way ANOVA	0,678
		Ham-Trad	One way ANOVA	0,127
		Ind-Trad	One way ANOVA	0,49

The statistical analysis of the matrix influence revealed that *P. nordicum* was matrix-sensitive, with growth being significantly higher in Ham. The strain of *P. nordicum* used in

this study has been isolated from ham, and maybe it is not adapted to sausage composition. On the other hand, *A. westerdijkiae*, although isolated from ham also, showed similar growth independently of the medium.

In terms of OTA production, the inability of *P. nordicum* to produce OTA in Ind found in the preliminary test was confirmed also for Trad. For *A. westerdijkiae* the matrix effect, although not significant, can also easily be observed for yeast and starter treatments. The production of OTA is always higher in Trad than in Ind, maybe due to the fact that industrial sausage probably has more anti-fungal preservatives added than traditional sausage. While yeasts enhanced OTA more strongly in Ham, starter showed stronger effect on sausage-based media. This difference may be a result of starter being more adapted to sausage than to ham matrix, since it is a culture commercially used in sausage fermentation.

To determine the real reasons behind this matrix effect, it would be necessary to determine and compare the chemical composition of these three meat products throughout the ripening period, which is out of the scope of this study. It would be very interesting to define the mechanism behind this effect because it could be used as a solution to prevent OTA production. Further studies are needed in this field of research.

4.2.3 Mechanisms of action

Mechanisms involved in the way microorganisms affect growth and OTA production ability of *P. nordicum* and *A. westerdijkiae* need to be clarified in order to optimise their applications in meat products. In fact, the inhibitor effect on one fungus and stimulation of the other one is disturbing and could obstruct the use of those microorganisms as biocontrol agents. The intention of this part of the study was to try to shed some light on the mechanisms underlying the effect of the test organisms on growth and OTA production by the two fungi. Based on the responses of fungi towards all microorganisms tested as biocontrol agents previously obtained, *C. zeylanoides* and starter culture were selected for further studies given the similarity of results obtained for Trad and Ind media, tests were run only in Ham and Trad. Since *R. mucilaginosa* showed limited ability to grow in Ham, it was also excluded from the following steps.

4.2.3.1 Starter culture

Results of fungal colony diameter of *P. nordicum* and *A. westerdijkiae*, as well as OTA production relative to different methods of co-culture with the starter, in Trad and Ham media with 3% NaCl incubated at 20 ºC are represented respectively in **figures 4.9** and **4.10.**

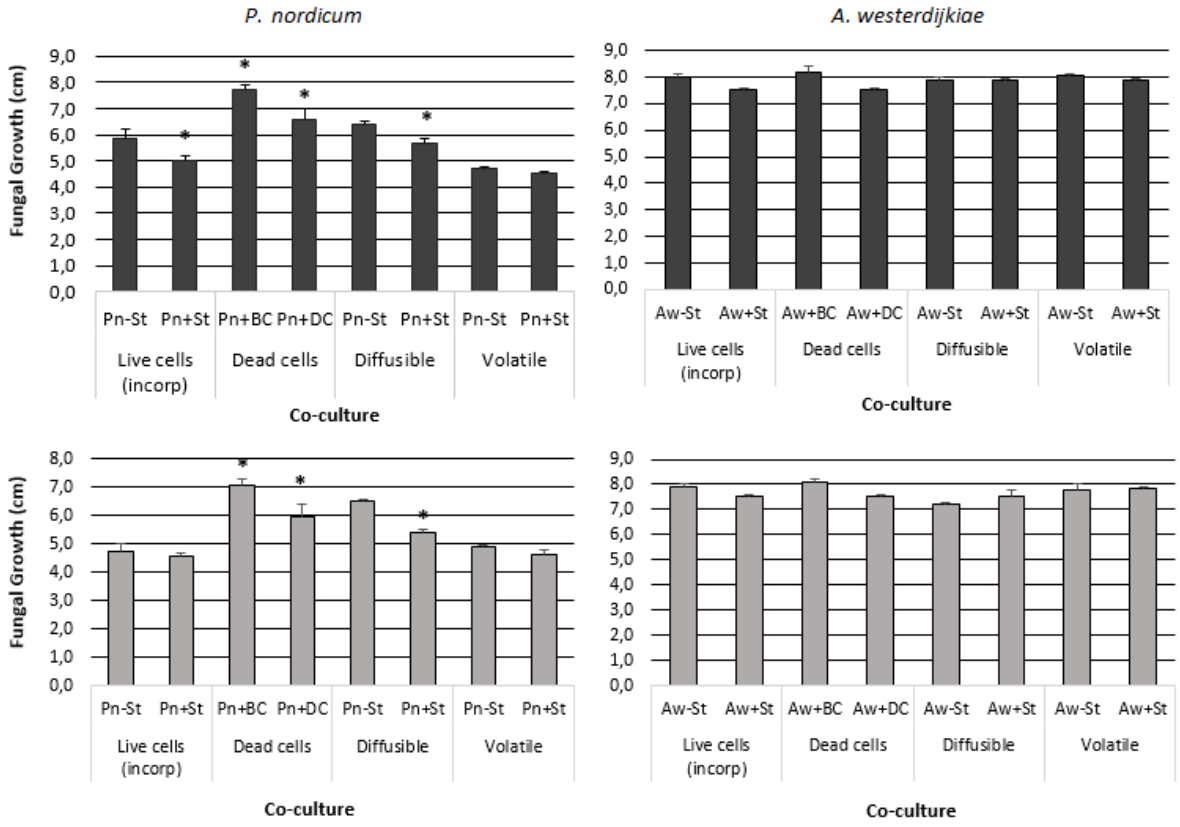


Figure 4.9 Growth of P. nordicum (left) and A. westerdijkiae (right) in two different media – Ham (top; black bars) and Traditional sausage (bottom; light bars) – at 20 ºC and 3% NaCl after 14 days of incubation. In all cases, results are the average of six replicates; standard deviation is indicated as vertical thin lines. The control for cell broth and of dead cells testes was Pn-St (incorp)
(Pn: *P. nordicum*; Aw: *A. westerdijkiae*; Fungus-St: control (fungus without starter); Fungus+St: fungus with incorporated starter; Fungus+BC: inoculation in the broth in which starter cells were grown; Fungus+DC: inoculation in medium with incorporated dead cells)
* Significantly different from control (fungus only), $p \leq 0.05$

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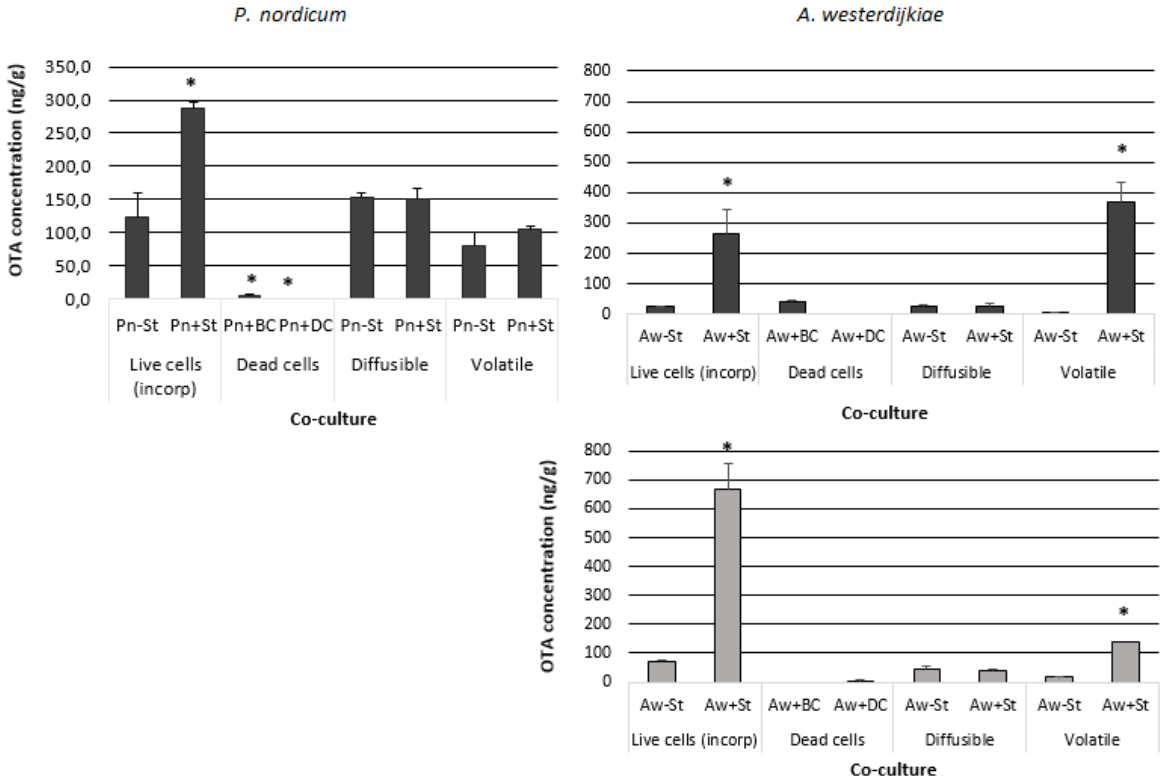

Figure 4.10 OTA production by *P. nordicum* (left) and *A. westerdijkiae* (right) in two different media – Ham (top; black bars) and Traditional sausage (bottom; light bars) – at 20 ºC and 3% NaCl after 14 days of incubation. In all cases, results are the average of six replicates; standard deviation is indicated as vertical thin lines. The control for cell broth and of dead cells tested was Pn-St (incorp)
(Pn: *P. nordicum*; Aw: *A. westerdijkiae*; Fungus-St: control (fungus without starter); Fungus+St: fungus with incorporated starter; Fungus+BC: inoculation in the broth in which starter cells were grown; Fungus+DC: inoculation in medium with incorporated dead cells)
* Significantly different from control (fungus only), $p \leq 0.05$

Results of *P. nordicum* growth showed that the incorporated live cells reduced fungal growth (significantly only in Ham), whereas cell broth and dead cells significantly stimulated fungal growth in both media. For *P. nordicum*, growth patterns are similar for both media suggesting that cell broth or a cell component can be exerting the stimulation effect. In respect to OTA production, *P. nordicum* produced detectable levels only in Ham medium, confirming previous results. While the incorporation of live cells significantly stimulated OTA production (as previously described**)**, a significant decrease was observed for both cell broth and incorporated dead cells (as compared to Pn-St). No significant effect was observed on OTA production in diffusible and volatile tests. As a matter of consequence, direct contact between the starter and fungus seems to have a significant effect. This can be a result of competitive exclusion, based on competition for nutrient and space (Zhao et al, 2008).

But, while for non-toxigenic organisms the result is usually reduced growth (as also happens for *P. nordicum*), for mycotoxigenic fungi this can mean induction of secondary metabolism and of mycotoxin production. In fact, fungal secondary metabolism is strongly stimulated by sub-optimal conditions like nutrient depletion or metabolites produced by the starter organisms. Since OTA is inhibited by cell broth and by dead cells we can assume that starter metabolites responsible for OTA induction are only produced by the starter as response to the presence of the fungus, and that on cell broth and on dead cells extra nutrients are being offered to the fungus, thus justifying increased growth and decreased OTA production.

In the case of *A. westerdijkiae* growth, no significant effect was detected between control and test conditions and also between treatments. On the other hand, a significant stimulation of OTA production in both Ham and Trad media occurred for the incorporation of live cells and for the volatile test. For this fungus, direct contact of broth organisms seems to have a significant effect, which is stronger in Trad. This could be explained by the fact that starter culture is more adapted to this matrix assuming higher or faster growth, and consequently stronger effect in this medium. The results obtained for volatile compounds are puzzling, since cells inoculated for the diffusible test would also produce the same type and concentration of volatiles. The fact that stimulation occurs only for the volatile test but not for the diffusible test needs to be further clarified. Cell both and dead cells, don´t seem to exert any type of effect on *A. westerdijkiae*.

4.2.3.2 *Candida zeylanoides*

The results of *P. nordicum* and *A. westerdijkiae* growth co-cultured with *C. zeylanoides* under different methods in Trad and Ham media with 3% NaCl at 20 ºC of incubated are represented in **figures 4.11** and **4.12**. The results of OTA production are represented in **figure 4.13**.

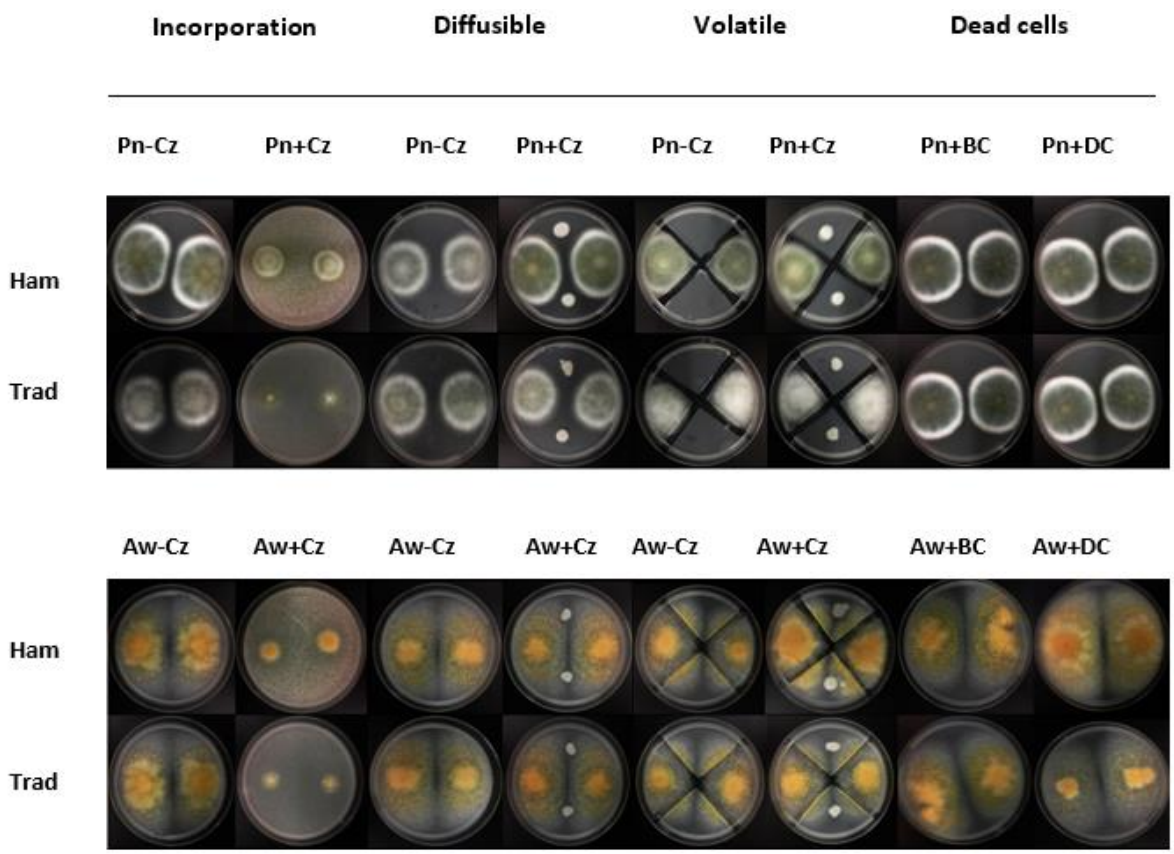

Figure 4.11 Growth of *P. nordicum* and *A. westerdijkiae* in Traditional and Ham-based media under different conditions of co-culture with C. zeylanoides at 20 ºC
(Pn: *P. nordicum*; Aw: *A. westerdijkiae*; Fungus-Cz: control (fungus without yeast); Fungus+Cz: fungus with incorporated yeast; Fungus+BC: inoculation in the broth in which yeast cells were grown; Fungus+DC: inoculation in medium with incorporated dead cells)

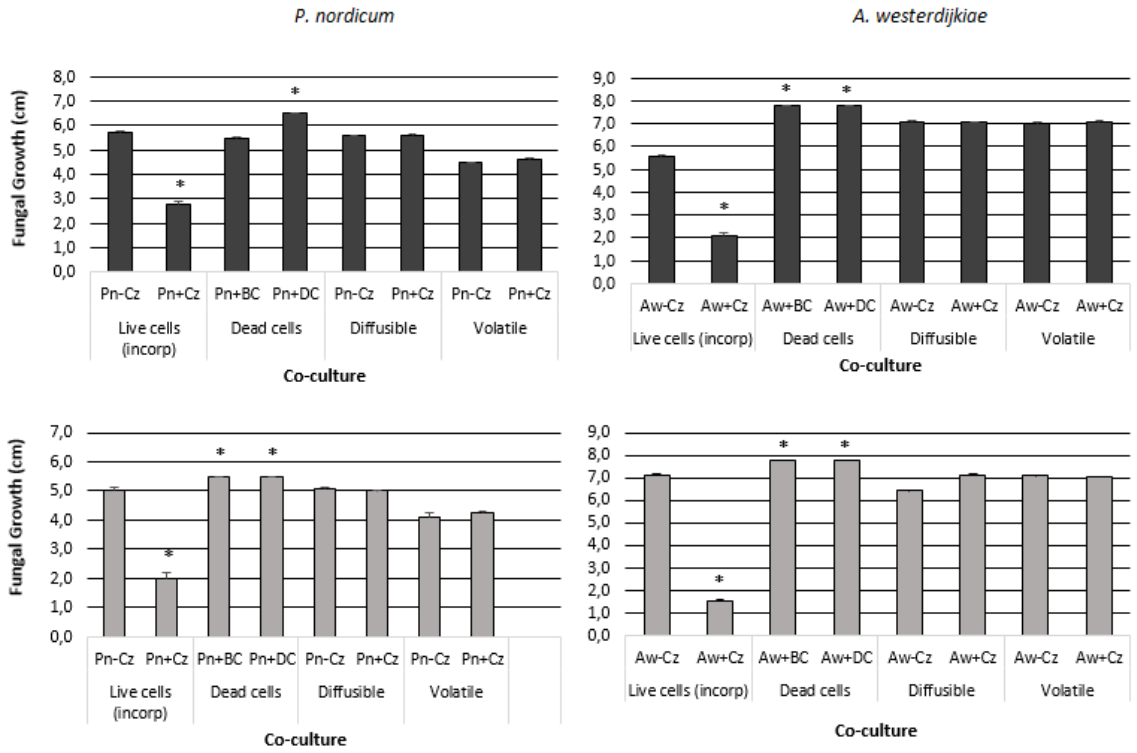

Figure 4.12 Growth of *P. nordicum* (left) and *A. westerdijkiae* (right) in two different media – Ham (top; black bars) and Traditional sausage (bottom; light bars) – at 20 ºC and 3% NaCl. In all cases, results are the average of six replicates; standard deviation is indicated as vertical thin lines
(Pn: *P. nordicum*; Aw: *A. westerdijkiae*; Fungus-Cz: control (fungus without yeast); Fungus+Cz: fungus with incorporated yeast; Fungus+BC: inoculation in the broth in which yeast cells were grown; Fungus+DC: inoculation in medium with incorporated dead cells)
* Significantly different from control (fungus only), $p \leq 0.05$

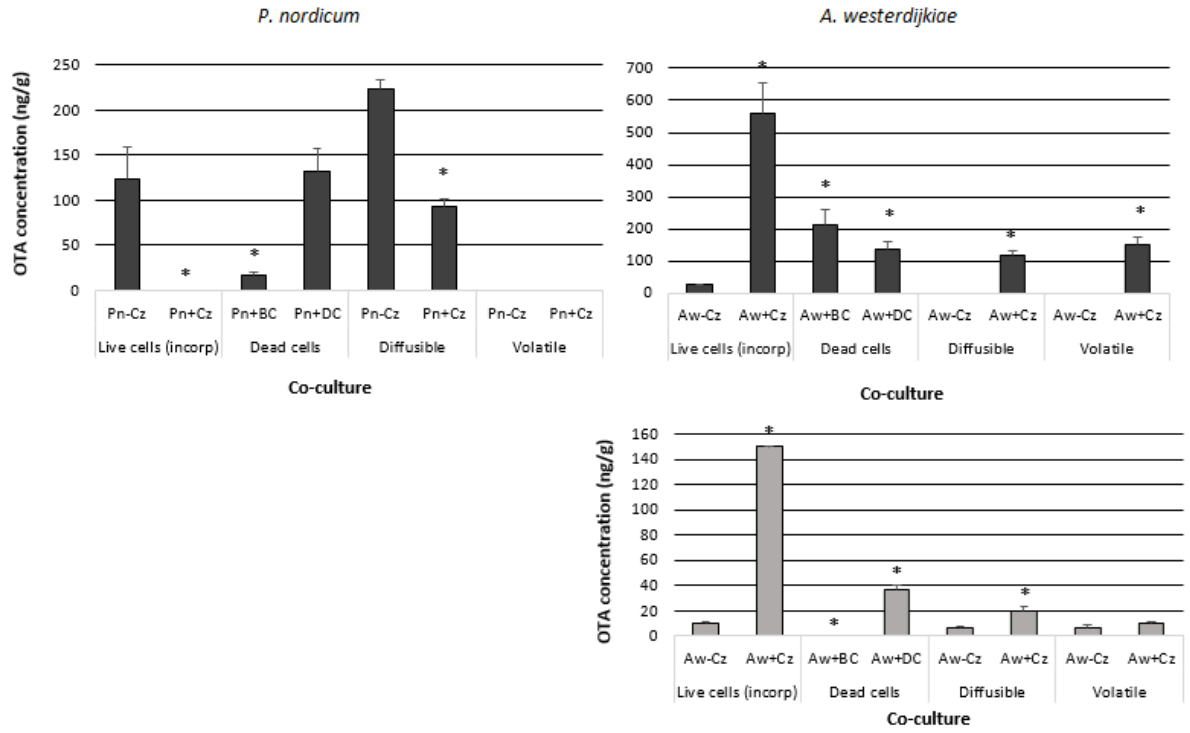

Figure 4.13 OTA production by *P. nordicum* (left) and *A. westerdijkiae* (right) in two different media – Ham (top; black bars) and Traditional sausage (bottom; light bars) – at 20 ºC and 3% NaCl. In all cases, results are the average of six replicates; standard deviation is indicated as vertical thin lines. The control for cell both and of dead cells testes was Pn-Cz (incorp).
(Pn: *P. nordicum*; Aw: *A. westerdijkiae*; Fungus-Cz: control (fungus without yeast); Fungus +St: fungus with incorporated yeast; Fungus+BC: inoculation in the broth in which yeast cells were grown; Fungus+DC: inoculation in medium with incorporated dead cells)
* Significantly different from control (fungus only), $p \leq 0.05$

C. zeylanoides had similar effect on growth of both *P. nordicum* and *A. westerdijkiae*. Growth data for both fungi for the incorporation test are in agreement with the results previously obtained (*cf.* section 4.2.2), where the incorporation of live cells significantly inhibited *P. nordicum* and *A. westerdijkiae* growth in Trad and Ham media. On the contrary, growth was generally increased with the incorporation of dead cells and of cell broth. These effects on growth are similar to those registered and discussed for the starter culture.

As previously demonstrated, *P. nordicum* does not produce OTA in Trad medium. However, in Ham it produced 123 ng/g. *C. zeylanoides* inhibited significantly OTA production by *P. nordicum* under three test conditions: incorporation of live cells, cell broth and diffusion. Since no OTA was detected in the control of volatile test, we cannot conclude about the effect of volatile compounds on OTA production. Contrary to the effect of the starter, where OTA was stimulated by living cells, *C. zeylanoides* inhibited both growth and OTA, and the effect

seems to be exerted by extrolites produced *C. zeylanoides*. Since the difference in OTA levels between incorporated cells and cell broth is not significant, it seems like the inhibiting effect results from extrolites produced by the yeast independently of direct contact between both organisms. These extrolites seem to be produced by the yeast and diffused towards the fungus. This effect is seen by the reduced amount of OTA in the diffusible test. For this test, the inhibitory effect is not as strong as the previously described, which leads to the conclusion that the extrolites are reaching the fungus at limited concentrations by diffusion throughout the medium.

For *A. westerdijkiae* strong and significant stimulation is observed by direct contact in both media. In Ham, *C. zeylanoides* seems to have cumulative effect of direct contact and diffusible compounds being produced in the medium independently of the presence of the fungus (seen by stimulation by cell broth), effect of cell structural compounds (determined by incorporation of dead cells) and volatiles. On the other hand, in Trad, low amounts of OTA are generally produced in all test conditions (< 38 ng/g) except for incorporation of live cells. Even though statistical analysis determines significant differences between tests, we consider that no test clearly justifies stimulation except for direct contact between *A. westerdijkiae* and *C. zeylanoides*.

6. CONCLUSIONS

In this work, we were able to reach the following conclusions:

a) *Penicillium nordicum* is strongly influenced by matrix. Even though this fungus is fully adapted to protein and salt-rich matrices like processed meat products, it was not able to produce OTA in sausage-based media, probably due to the chemical composition of this matrix (preservatives, condiments, etc). In this case, it would be of major interest to study chemical compounds from these matrices that could be responsible for this effect, and thus be used as OTA control agents;

b) *Aspergillus westerdijkiae*, although not generally associated with meat products, was not significantly influenced by the matrix, and showed to be capable of producing high amounts of OTA in these products. It has been demonstrated that this fungus should also be subject of interest in future works dealing with OTA contamination of meat products;

c) Yeasts were able to inhibit *P. nordicum* ability to produce OTA in ham, but unexpectedly OTA production was strongly stimulated by the starter culture. In *A. westerdijkiae*, yeasts and starter significantly enhanced OTA production;

d) Fungal growth and OTA production seem to be independent factors, since OTA was stimulated in several, but not all, cases where growth was inhibited;

e) Direct contact and simultaneous growth of test organisms were the mechanisms more significantly involved in fungal responses;

f) Yeasts and starter culture used in this study showed different effects on the two ochatoxigenic fungi, which means that the study of biocontrol agents against mycotoxigenic fungi must involve the microbial community instead of isolated microorganisms.

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