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Biocontrol of Ochratoxigenic moulds (*Aspergillus ochraceus* and *Penicillium nordicum*) by *Debaryomyces hansenii* and *Saccharomycopsis fibuligera* during speck production.

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Abstract

Speck is a meat product obtained from the deboned leg of pork that is salted, smoked and seasoned for four to six months. During speck seasoning, *Eurotium rubrum* and *Penicillium solitum* grow on the surface and collaborate with other moulds and tissue enzymes to produce the typical aroma. Both of these strains usually predominate over other moulds. However, moulds producing ochratoxins, such as *Aspergillus ochraceus* and *Penicillium nordicum*, can also co-grow on speck and produce ochratoxin A (OTA). Consequently, speck could represent a potential health risk for consumers. Because *A. ochraceus* and *P. nordicum* could represent a problem for artisanal speck production, the aim of this study was to inhibit these mould strains using *Debaryomyces hansenii* and *Saccharomycopsis fibuligera*. Six *D. hansenii* and six *S. fibuligera* strains were tested *in vitro* to inhibit *A. ochraceus* and *P. nordicum*. The *D. hansenii* DIAL 1 and *S. fibuligera* DIAL 3 strains demonstrated the highest inhibitory activity and were selected for *in vivo* tests. The strains were co-inoculated on fresh meat cuts for speck production with both of the OTA-producing moulds prior to drying and seasoning. At the end of seasoning (six months), OTA was not detected in the speck treated with both yeast strains. Because the yeasts did not adversely affect the speck odour or flavour, the strains are proposed as starters for the inhibition of ochratoxigenic moulds.

1. Introduction

Speck is a typical meat product in the European alpine area, particularly North Tyrol (Austria) and South Tyrol (Alto Adige, Italy), regions that utilize different recipes but the same technology. A flow chart showing the traditional speck production process is presented in Table 1. Fresh meat cuts (FMC) obtained from deboned legs are carefully brined with a mixture of salt and spices, such as pepper, juniper, laurel and rosemary, and the percentages of these ingredients depend on the facility. The meat cuts are then cold smoked (20 °C), dehydrated and seasoned for four to six months at 10-15 °C with a relative humidity (R.H.) between 60 and 90%. During this period, moulds grow on the meat surface and give it a distinctive flavour. The colonization of speck during drying and seasoning occurs spontaneously from the mycobiota of the production environment or by contamination with spices (Peintner et al., 2000). The indigenous mycobiota are valued for their beneficial seasoning effects (Peintner et al., 2000). Moulds prevent excessive drying of the surface, improve the texture, and limit excessive hardness of the flesh due to their proteolytic activity (Sunesen and Stahnke, 2003). They also exerts an antioxidant effect, contribute to colour production (Spotti et al., 2008) and improve the aroma and flavour of speck (Rojas et al., 1991, Nunez et al., 1996). The surface moulds of meat products have been studied in great detail (Dragoni et al., 1980; Huerta et al., 1987; Spotti et al., 1989; Rojas et al., 1991; Nunez et al., 1996; Battilani et al., 2007; Iacumin et al., 2011). *Eurotium* spp. appears to be the most prevalent during all stages of meat production (Peintner et al., 2000; Comi et al., 2004; Battilani et al., 2010; Comi and Iacumin, 2013). In North and South Tyrol, *Eurotium rubrum* and *Penicillium solitum* are the dominant species in all parts of speck (crust, meat and fat). Other Pennicillia, including *P. nalgiovense, P. verrucosum, P. canescens, P. brevicompactum, P. chrysogenum, P. glabrum, P. commune* and *P. waksmanii*, are less frequently observed (Peintner et al., 2000). The development of mould can also be related to negative effects, such as the production of mycotoxin (Comi and Iacumin, 2013). Several recent investigations have highlighted the presence of

ochratoxin A (OTA) in different meat products, and several fungi, including *Penicillium verrucosum, P. nordicum,* and *Aspergillus ochraceus*, can produce OTA following growth on the surface of meat products during seasoning and storage (Matrella et al. 2006; Pietri et al. 2006; Iacumin et al., 2011). OTA is undesirable because it is classified by IARC (International Agency for Research of Cancer) into "Group B" as a molecule with possible carcinogenic activity in humans (IARC, 1993). For this reason, limits have been introduced to foods. In Italy, the maximum concentration of OTA allowed in meat and meat products is 1 µg/kg (Ministry of Health Circular no. 10-93 09/06/1999). Different artisanal production lots of speck were recently contaminated with *A. ochraceus* and *P. nordicum*. Both species grow during drying and seasoning and produce up to 1 µg/kg OTA. Many technologies must be used to eliminate the growth of OTA-producing moulds on meat products. The most interesting methods developed to date are represented by bioprotective cultures. Exploring the antagonistic activity of autochthonous yeast strains against moulds could be of great interest. Several researchers (Andrade et al., 2014; Virgili et al., 2012) have experimented with the ability of native yeasts to protect meat products against mould growth. *D. hansenii* is the main yeast present in meat products from the early stage of seasoning to the end, when it becomes the predominant strain (Comi and Cantoni, 1980; Nunez et al., 1996; Asefa et al., 2010; Simoncini et al., 2007). Consequently, *D. hansenii* is largely used as a starter culture to improve the quality and sensorial characteristics of European sausages (Hammes and Knauf, 1994). Conversely, *S. fibuligera* is rarely present in meat products because it is typical of starchy substrates and vegetables (Chi et al., 2009). However, it is used because it grows in the presence of high salt and sugar concentrations similarly to *D. hansenii*. Because yeasts are capable of growing and colonizing meat products, they could represent the main strains for food biocontrol against moulds and pathogenic microorganisms (Virgili et al., 2012; Andrade et al., 2014; Simoncini et al., 2014). The efficacy of the protection provided by yeasts against moulds

on meat products must be seriously evaluated because moulds often grow on meat product surfaces despite the presence of yeasts (Virgili et al., 2012). Little information regarding the antagonistic activity of yeasts on dry cured ham has been reported (Virgili et al., 2012), and different researchers have recently investigated and demonstrated the inhibitory effect of yeasts *in vitro* against OTA-producing moulds (Andrade et al., 2014; Simoncini et al., 2014). These researchers have demonstrated that it is possible to reduce *P. nordicum* growth and activity using different yeast strains. The inhibitory mechanism is not well understood, but it has been suggested that yeasts restrict nutrient availability and sites for colonization and consequently predominate and limit the growth of OTA-producing moulds (Björnberg and Schnürer, 1993; Spotti et al., 2009). Considering the many opportunities that yeasts can have in mould inhibition, this study aimed to evaluate the abilities of *Debaryomyces hansenii* and *Saccharomycopsis fibuligera* to control ochratoxigenic mould growth (*Aspergillus ochraceus* and *Penicillium nordicum*) during speck production.

2. Materials and methods

Six strains of *Saccharomycopsis fibuligera* (DIAL 1, DIAL 2, DIAL 3, DIAL 4, DIAL 5, and DIAL 6) and six strains of *Debaryomyces hansenii* (DIAL 1, DIAL 2, DIAL 3, DIAL 4, DIAL 5, and DIAL 6) were selected from a total of 90 isolates from meat products and stored in the Collection of the Department of Food Science of the University of Udine (DIAL, Udine, Italy). The twelve yeast strains were selected for potential competition against *Penicillium nordicum* and *Aspergillus ochraceus*. The strains were maintained in malt extract modified agar (MEMA, Oxoid, Italy) supplemented with dextrose (1%) and peptone (1%).

2.1. Preparation of yeast inocula

The yeast strains were grown on MEMA at 25 °C for 48 h. Suspensions were then prepared by adding a loop full of cells to peptonised water (0.7% NaCl and 0.1% peptone in 1.000 mL of water). The density of the yeast cultures was determined spectrophotometrically by measuring their optical density at 600 nm. Serial dilutions were prepared to obtain the concentration used for the experiments.

2.2. Preparation of the P. nordicum and A. ochraceus inocula

One *P. nordicum* and one *A. ochraceus* strain were used in this study. These strains were isolated from sausages in a previous study and identified at the molecular level. Both strains produce OTA and belong to the Food Science Department of the University of Udine. Both strains were grown in Czapek yeast extract agar plates (Oxoid, Italy) and incubated at 25 °C for seven days. At the end of the incubation period, the conidia were removed from the culture surface according to the method reported by Virgili et al. (2012). The conidial suspension was adjusted to 10^2, 10^4 and 10^6 conidia/mL using a haemocytometer.

2.3. Inhibition of P. nordicum and A. ochraceus by yeast strains

The assay was performed using the method described by Virgili et al. (2012) and Bleve et al. (2006) and modified as follows: the experiments were performed on MEMA adjusted to pH 6.0. A top agar was prepared by mixing 10 mL of MEMA broth with 0.7% agar and 1 mL of a yeast suspension (*D. hansenii* or *S. fibuligera*) containing 10^6 CFU/mL to obtain a thick, continuous layer on the plate surface. This suspension was distributed into Petri plates containing 15 mL of MEMA. Three 10 µL portions of *P. nordicum* or *A. ochraceus* corresponding to 10^6 conidia/mL were then spotted onto each plate and incubated at 20 °C. Top agar containing different yeast concentrations (10^2, 10^4, or 10^6 CFU/mL) was also prepared to investigate differences among different yeast inocula.

Three replicate experiments were performed for each yeast. Plates with culture medium (MEMA) that were not inoculated with yeast were included as controls.

Fungal growth was expressed as the average measurement (mm) of two orthogonal diameters per colony after 14 days of incubation at 20 °C. The inhibitory activity was calculated using the equation reported by Lima et al. (1999):

$$\% \text{ Inhibitory activity} = \frac{(\text{Fungal growth in control plate} - \text{Fungal growth in treated plate}) \times 100}{\text{Fungal growth in control plate}}$$

2.4. Inhibitory activity at different concentrations of the selected yeasts

The above-mentioned method (Virgili et al., 2012) was used the following modifications, and the concentration of yeast in top agar was changed. Briefly, top agars containing different concentrations of the yeasts (10^2, 10^4, or 10^6 CFU/mL) were used to examine differences among yeast inocula concentrations. *D. hansenii* (strain DIAL 1) and *S. fibuligera* (DIAL 3), which demonstrated the best performance in the above-mentioned test, were used. Ten millilitres of inoculated top agar were distributed onto 15 mL of MEMA, and three 10-µL suspensions containing 10^6 conidia/mL of the moulds were then spotted separately onto each plate and incubated at 20 °C for 14 days. Three replicate experiments were performed for each yeast concentration. MEMA plates that were not inoculated with yeast were included as controls.

2.5. Inhibitory activity at different concentrations of moulds (P. nordicum and *A. ochraceus)*

The above-mentioned method (Virgili et al., 2012) was used with the following modifications. The concentration of yeasts in top agar was 10^4 CFU/mL, and *D. hansenii* (strain DIAL 1) and *S. fibuligera* (DIAL 3), which demonstrated the best performance in the above-described test, were used. Ten millilitres of inoculated top agar were distributed

onto 15 mL of MEMA, and three 10-µL suspensions containing different concentrations of conidia (10^2, 10^4, or 10^6 conidia/mL) of both moulds were then separately spotted onto each plate and incubated at 20 °C for 14 days. Three replicate experiments were performed for each mould concentration. MEMA plates that were not inoculated with yeast were included as controls.

2.6. Inhibitory activity of yeasts on OTA production in the speck model system (Battilani et al., 2010, modified)

Five specks with different water activity (Aw) values were collected from a South Tyrolean facility. From each speck, samples (50 mm in diameter and 5 mm in height) were excised using a hollow metal sampler with a cylindrical cutting edge. The Aw values of the samples measured with an AquaLab CX-2 instrument (Steroglass, Pullman, WA, USA) were 0.960 ± 0.005, 0.940 ± 0.005, 0.920 ± 0.005, 0.900 ± 0.005, and 0.880 ± 0.005. After dipping in absolute ethanol for 2 min, the samples were removed and flamed to sterilize them prior to inoculation (Rojas et al., 1991). The samples were placed in Petri plates, inoculated and placed in sealed boxes equipped with beakers containing NaCl solutions (Multon and Bizot, 1980) with the same Aw as the five speck samples. The salt solutions were prepared with distilled water (w/w) with 6.57% NaCl (Aw 0.96), 9.38% NaCl (Aw 0.94), 11.90% NaCl (Aw 0.92), 14.18% NaCl (Aw 0.90) and 16.28% NaCl (Aw 0.88). Each Aw value was confirmed using AquaLab CX-2.

The samples were incubated in the dark at 14 °C for 30 days. Six different inocula or co-inocula were tested: 1) Control A, 10^4 conidia/cm^2 *Aspergillus ochraceus*; 2) Control B, 10^4 conidia/cm^2 *Penicillium nordicum*; 3) 10^6 CFU/g *D. hansenii* DIAL 1 vs. 10^4 conidia/cm^2 *Aspergillus ochraceus*; 4) 10^6 CFU/g *D. hansenii* DIAL 1 vs. 10^4 conidia/cm^2 *Penicillium nordicum*; 5) 10^6 CFU/g *S. fibuligera* DIAL 3 vs. 10^4 conidia/cm^2 *Aspergillus ochraceus*; and 6) 10^6 CFU/g *S. fibuligera* DIAL 3 vs. 10^4 conidia/cm^2 *P. nordicum.* Each

condition was evaluated in triplicate. At the end of the incubation period, the 90 samples were collected and analysed for OTA according to the method reported by Matrella et al. (2006).

2.7. Inhibitory activity of yeasts on OTA production during speck seasoning

One hundred and twenty fresh meat cuts (FMCs) were collected. Each FMC (6.5 kg) was trimmed and squared according to the traditional procedure and was then salted and smoked. After smoking, the FMCs were inoculated with a mix of yeast and mould suspensions or with a mould suspension alone according to the following experimental design: 20 FMCs were inoculated with a suspension of *P. nordicum* at a final concentration 10^4 conidia/cm^2; 20 FMCs were inoculated with a suspension of *A. ochraceus* at a final concentration 10^4 conidia/cm^2; 20 FMCs were inoculated with a mix of *A. ochraceus* and *D. hansenii* (final concentration 10^4 conidia/10^6 CFU/cm^2); 20 FMCs were inoculated with a mix of *A. ochraceus* and *S. fibuligera* (10^4 conidia/10^6 CFU/cm^2); 20 FMCs were inoculated with a mix of P. *nordicum* and *D. hansenii* (10^4 conidia/10^6 CFU/cm^2); and 20 FMCs were inoculated with a mix of *P. nordicum* and *S. fibuligera* (10^4 conidia/10^6 CFU/cm^2). The *S. fibuligera* DIAL 3 and *D. hansenii* DIAL 1 strains were used. All of the FMCs were seasoned for six months according to the traditional procedure (Table 1). At the end of the seasoning period, 100-cm^2 samples were taken from a depth of 0.5 cm below the speck surface and analysed for OTA. Briefly, meat collected from a depth of 0.5 cm below the slime was homogenized using a Stomacher instrument (Lab Blender 400, PBI, Italy), and 10 g of the homogenate was then used for the analysis. OTA was extracted and evaluated according to the method described by Matrella et al. (2006). Before sampling for OTA analysis, the surface of each speck was observed with a Stereoscope (320X; WILD M 420, Heerbrugg, CH) to determine the presence of mould growth (i.e., hyphae).

232 *2.8. Sensorial analysis*

233 To evaluate the influence of the yeast culture starter on the organoleptic characteristics of
234 the product, a sensory analysis was performed using the triangle test methodology (ISO
235 4120:2004). FMCs were divided into three lots. After smoking, lot A (10 cuts) was
236 inoculated with a suspension of *S. fibuligera* DIAL 3 (10_6 CFU/cm_2), and Lot B (10 cuts)
237 was inoculated with *D. hansenii* DIAL 1 (10_6 CFU/cm_2). Lot C (10 cuts) served as the
238 control and was not inoculated with yeast cultures. All cuts were seasoned according to the
239 traditional procedure (Table 1). At the end of seasoning (6 months), all of the lots were
240 subjected to the triangle test, which was used to compare Lot A to Lot B, Lot A to Lot C,
241 and Lot B to Lot C. Twenty non-professional assessors were presented with three products,
242 two of which were identical. The assessors were asked to state which product they believed
243 was a unique sample. The assessors who indicated the existence of two distinct samples
244 were asked to identify the best sample.

245

246 *2.9. Statistical analysis*

247 The values of the various parameters were compared through one-way analysis of
248 variance. The averages were compared with Tukey's honest significance test using the
249 Statistical Graphics software package (Rockville, MD, USA).

250

251 **3. Results**

252 A biocontrol test was performed using six *Saccharomycopsis fibuligera* and six
253 *Debaryomyces hansenii* strains isolated from meat products. Four out of the six *D.*
254 *hansenii* and three out of the six *S. fibuligera* strains were detected in dry cured ham. The
255 other strains were isolated from sausages. All of the strains were tested for their ability to
256 grow *in vitro* and *in vivo* in the presence of low Aw (0.88) and 6% NaCl and on speck and
257 dry cured ham (data not shown).

Different *in vitro* inhibitory activities against *P. nordicum* and *A. ochraceus* were observed among the tested strains ($p < 0.05$). *D. hansenii* DIAL 1 and *S. fibuligera* DIAL 3 showed the highest inhibitory activities against *A. ochraceus* (76.0% and 86.1%, respectively) and *P. nordicum* (78.8% and 85.2%, respectively; Tables 2-3). The diameters of *A. ochraceus* colonies were reduced from 32.5 mm (control) to 4.5 mm by *S. fibuligera* and to 7.8 mm by *D. hansenii*. The diameters of *P. nordicum* were reduced from 20.3 mm (control) to 3.0 and 4.3 mm by *S. fibuligera* and *D. hansenii*, respectively.

For this reason, both strains were selected for the assessment of their *in vitro* or *in vivo* inhibitory effects. Different concentrations of both yeast strains were tested separately to identify the best strain. As expected, the inhibitory activity was dependent on the yeast concentration. Indeed, an *in vitro* concentration of 10_6 CFU/mL of agar showed the best inhibitory effect for both of the tested yeasts ($p < 0.05$; Table 4). The antagonistic activities of the yeasts (10_4 CFU/mL) were evaluated separately against different concentrations of both moulds (10_2, 10_4, and 10_6 conidia/mL). The percentage of inhibition varied according to the mould concentration (Table 5). The lowest percentage of inhibition was observed after inoculation with the highest mould concentration ($p < 0.05$). The activity of *S. fibuligera* was higher than the activity of *D. hansenii*. A higher yeast concentration (i.e., 10_6 CFU/mL) completely inhibited mould at concentrations of 10_2 and 10_4 conidia/mL (data not shown), and the same results, which are shown in Table 4, were obtained with similar yeast and mould concentrations ($10_6/10_6$ CFU/mL). The measured diameters demonstrated that the inhibitory activity of *S. fibuligera* was higher than that of *D. hansenii* ($p < 0.05$), even though the media used appeared to be more productive for *D. hansenii* than for *S. fibuligera.*

A reduction in fungal growth and consequently a decrease in OTA production were observed in speck model systems (Photograph 1). Table 6 displays the effects of the yeast strains on the OTA production of the tested moulds in pieces of speck with different Aw

284 levels. As shown, a low OTA concentration was detected in the pieces of speck inoculated
285 with both yeast and mould strains. The OTA concentration in the speck co-inoculated with
286 yeast and mould strains was less than 1 µg/kg (Table 6), which is the limit allowed in meat
287 and meat products by the Italian Ministry of Health (Circular no. 10-93 09/06/1999). In
288 contrast, OTA concentrations in speck inoculated only with mould reached 1 µg/kg. As
289 expected, pieces of speck with the higher Aw values (i.e., 0.96 and 0.94) exhibited the
290 highest OTA concentrations (Table 6).
291 The inhibitory activity of both yeasts against OTA-producing moulds during speck
292 production was tested *in vivo*. Both yeasts were co-inoculated separately in FMCs with the
293 *A. ochraceus* and *P. nordicum* strains. The strains inhibited both of the moulds *in vivo*. An
294 inoculum with the tested strains was prepared after the smoking phase. Less than 0.1 µg/kg
295 OTA was detected in speck inoculated with yeast and mould (Table 7). Conversely, OTA
296 concentrations of 65.5 µg/kg and 68.1 µg/kg were detected in speck inoculated with *A.*
297 *ochraceus* and *P. nordicum*, respectively. No *hyphae* or OTA-producing moulds were
298 observed on the surface of speck using a stereoscope, as demonstrated in Photograph 2.2.
299 The data demonstrated that both of the tested yeast strains were able to reduce or
300 completely inhibit the growth of *A. ochraceus* and *P. nordicum* both *in vitro* and *in vivo*.
301 Consequently, the yeasts can inhibit OTA production.
302 Based on the promising results obtained, the use of starter cultures of yeast to control
303 OTA-producing moulds might be recommended. However, it was necessary to evaluate the
304 influence of the starter yeasts on the acceptability of speck prior to implementing this
305 suggestion. Speck produced with and without the yeast starter were seasoned using the
306 traditional method (Table 1). At the end of seasoning, the speck samples were subjected to
307 a triangular test by a panel of 20 non-professional assessors. The entire panel confirmed
308 the acceptability of the speck supplemented with the starter cultures and found no
309 difference among Lots A, B (with bio-protective cultures) and C (without protective

cultures). The tasted speck slices were compact and homogeneous. The lean part was ruby red in colour, and the colour of the fat was white, which is typical of the product. The consistency was compact but not elastic, and the bouquet was delicate and distinctive. The taste was delicate, and there was no perception of spices or flavours.

4. Discussion

The potential biopreservative activities of six different *D. hansenii* and six *S. fibuligera* strains isolated from meat products against ochratoxigenic moulds were investigated in the present study. These species were selected because they are widespread on meat and meat products (Cocolin et al., 2006; Andrade et al., 2009) and are considered safe by the food industry (EFSA, 2012).

Speck is a typical meat product of North Tyrol (Austria) and South Tyrol (Alto Adige, Italy). In Italy, speck is also produced in the Veneto and Friuli Venezia Giulia regions. During the seasoning process, a large population of microorganisms (mainly moulds and yeasts) develops on the outer layers of the speck (Peintner et al., 2000). Some of these moulds could produce OTA, such as *P. nordicum* and *A. ochraceus*. Under some environmental conditions, their growth and ability to synthesize OTA could represent a real risk for human health. OTA has been found in different meats and meat products contaminated by ochratoxigenic moulds (Rojas et al., 1991; Battilani et al., 2010; Dall'Asta et al., 2010; Iacumin et al., 2011). However, the presence of OTA is not always correlated with OTA-producing moulds in meat products (Rodríguez et al., 2012).

Different methods can be used to inhibit OTA-producing moulds (Iacumin et al., 2011; Comi et al., 2013). The most commonly used method consists of spreading starter cultures on the meat product surface in the late drying and seasoning stages (Comi et al., 2013). In sausage production, mould and yeast starters are mainly used because they limit the growth of pathogenic microorganisms and because they improve the sensorial quality of the

product. Our study focused on the use of two yeast strains, and their activities were evaluated either *in vitro* or *in vivo*. Under *in vitro* conditions, *D. hansenii* and *S. fibuligera* significantly inhibited the growth of the tested ochratoxigenic moulds, and differences were observed among the *D. hansenii* and *S. fibuligera* strains. *D. hansenii* DIAL 1 and *S. fibuligera* DIAL 3 showed the highest efficiencies for the inhibition of *P. nordicum* and *A. ochraceus* on solid media.

The inhibitory effect of *S. fibuligera* DIAL 3 against both of the mould strains was higher and significantly different ($p < 0.05$) compared with the effect of *D. hansenii* DIAL 1. The inhibitory activity of *D. hansenii* strains against *P. nordicum* was also found to be reduced by Virgili et al. (2012), which is in agreement with the results of our investigation. These researchers found that two strains, which were selected for their ability to grow in dry cured ham-like substrates (*Candida zeylanoides* and *Hyphopichia burtonii*), were more effective against *P. nordicum* than against *D. hansenii* and *Candida famata* (the anamorphic form of *D. hansenii*). Indeed, both of these strains inhibited *P. nordicum* growth and OTA production (Virgili et al., 2012). Differences in inhibitory activity against *P. nordicum* were also found among *D. hansenii* strains among Andrade et al. (2014). These researchers demonstrated that the *in vitro* efficiency depends on the strains, the medium and the Aw value and that the lysed yeast cells might provide nutrients for *P. nordicum* growth. These researchers concluded that the inhibition observed appears to be due to compounds produced by yeast strains with activity against the tested moulds. Several researchers (Marquina et al., 2001; Masoud et al., 2005) have demonstrated that *D. hansenii*, *Saccharomyces cerevisiae* and *Pichia anomala* produce killer proteins against sensitive strains of yeasts and moulds. Again, the observed mould inhibition could be due to the competition activity of nutrients, as previously suggested (Björnberg and Schnürer, 1993; Zhao et al., 2008); however, researchers have speculated the existence of a minor effect on the production of extracellular volatile compounds and killer proteins.

362 Due to the large variability in OTA-producing moulds on speck and meat products (Virgili
363 et al., 2012; Asefa et al., 2010), yeasts inoculated with a lower concentration were
364 associated with a lower level of mould inhibition. Additionally, the *in vitro* inhibitory
365 activity of *S. fibuligera* against OTA-producing moulds was higher than the inhibitory
366 activity of *D. hansenii*. The use of different yeast concentrations *in vitro* resulted in
367 different percentages of mould inhibition, demonstrating that mould inhibition depends on
368 the competition for the nutrients but not on antimycotic production. Consequently, the
369 highest yeast concentration (10_6 CFU/g) induced the largest inhibitory action against both
370 of the OTA-producing moulds. The experiment confirmed the potential biocontrol effect of
371 both of the yeast strains used. Our data agree with the results reported by Virgili et al.
372 (2012). In fact, the inhibitory activity of yeast was affected by the mould concentration.
373 Virgili et al. (2012) hypothesized that the concentration of OTA-producing moulds in meat
374 products is a key factor for the effectiveness of yeast starters. Consequently, the meat
375 production industry should apply the most severe controls during the seasoning process to
376 limit the contamination and growth of OTA-producing moulds. Additionally, the raw
377 material, temperatures, and R.H. of the drying and seasoning rooms should be controlled.
378 In the speck model system, the data demonstrated the possibility of the effective biocontrol
379 of the OTA-producing moulds by the yeast. The results did not appear to depend on the
380 Aw, the yeasts or the mould strains. The reduced OTA concentration in the speck model
381 system with different Aw values compared with the control samples was quite obvious.
382 The antagonistic activities of *D. hansenii* and *S. fibuligera* were not dependent on the Aw
383 and were similar to data reported by Simoncini et al. (2014), who demonstrated that the
384 variability in the antagonistic effect was affected by the strains of the different species used
385 (*D. hansenii* and *C. zeylanoides*). A positive effect of both of the yeasts tested was
386 observed on the inhibition of mould growth, OTA production and accumulation. However,
387 no association was found between the presence of mycotoxins and the biomass of the

388 OTA-producing moulds. As demonstrated by Xu et al. (2007), the production and
389 concentration of some mycotoxins in food are not necessarily proportional to the biomass
390 of the OTA-producing mould. The biocontrol activity of *D. hansenii* has been
391 demonstrated by various researchers (Hernández-Montiel et al., 2010; Virgili et al., 2012;
392 Andrade et al., 2014). There are no data on the biocontrol activity of *S. fibuligera* against
393 OTA-producing moulds, even though different yeast species isolated from cheese, milk,
394 grape must, wine, fruits and vegetables have been demonstrated to inhibit many
395 mycotoxigenic moulds and to reduce the presence of mycotoxins (Bleve et al., 2006; Zhao
396 et al., 2008; Virgili et al., 2012; Simoncini et al., 2014). Thus, this study provides the first
397 demonstration of the use of this yeast strain for the biocontrol of OTA-producing moulds.
398 In addition, this study used *D. hansenii* DIAL 1 and *S. fibuligera* DIAL 3 as starter cultures
399 for speck production to eliminate the growth of OTA-producing moulds and to eliminate or
400 reduce the presence of OTA. OTA was found at a high level (>1 μg/kg) only in the speck
401 produced without the addition of yeasts. Inoculation of the FMCs with either *A. ochraceus*
402 or *P. nordicum* resulted in the production of OTA at a level that was higher than the limit
403 proposed by the Italian Ministry of Health (1 μg/kg). In the absence of yeasts, both of the
404 inoculated moulds grew on the FMCs from the late phase of drying to the end of the
405 seasoning phase. OTA production was detected in the meat. *P. nordicum* produced a white-
406 and-green cottoned slime (Photograph 2.1), whereas *A. ochraceus* produced a yellow
407 cottoned slime. Both cottoned slimes entirely covered the meat portion of the FMCs, but
408 no growth was observed on the skin of the FMCs. The co-inoculation of yeasts and moulds
409 did not permit mould growth on the FMCs. Therefore, the dominance of the inoculated
410 yeasts was the main parameter involved in the elimination of the growth of OTA-
411 producing moulds and consequently the elimination of OTA presence and production. In
412 the co-inoculated FMCs at the end of the seasoning phase, the amount of OTA was less
413 than the limit of detection of the method (< 0.1 μg/kg). The speck obtained with the starter

414 yeasts were safe and in accordance with the OTA limit proposed by the Italian Ministry of
415 Health (Circolare Ministero Sanità No. 30 10-09/06/1999).
416 Several researchers (Virgili et al., 2012; Andrade et al., 2014; Simoncini et al., 2014) have
417 demonstrated that inoculated and native yeasts are able to dominate P. *nordicum* and OTA-
418 producing moulds. Specifically, the antagonistic effects of some strains, such as *D.*
419 *hansenii, C. zeylanoides* and *H. burtonii,* are independent of the Aw, temperature and R.H.
420 However, these researchers recognized the existence of an antagonistic variability at the
421 level of the tested strains. Therefore, it was necessary to select the strain with the highest
422 antagonistic effect among the isolated species. This finding was confirmed in our study.
423 The significant absence of OTA appeared to be related to the inhibition of the growth of
424 both mould strains by the co-inoculated yeasts. No other hypotheses might be formulated.
425 In fact, no mould hyphae were observed on the surfaces of the co-inoculated specks under
426 a stereomicroscope. For this reason, the possibility that the absence of OTA in the co-
427 cultured samples might be due to mycotoxin degradation by the yeasts into less toxic
428 compounds was excluded (Andrade et al., 2014; Simoncini et al., 2014). Additionally, the
429 possibility that either of the yeasts could have adsorbed OTA on their cell wall was
430 excluded. Various researchers (Gil-Serna et al., 2011; Shetty et al., 2007) recently reported
431 that *S. cerevisiae* and *D. hansenii* could influence the regulation of mycotoxin
432 biosynthesis. The mechanisms involved in the reduction of OTA in the presence of yeasts
433 need to be clarified (Andrade et al., 2014). However, the results obtained by the
434 stereoscope observations made in this work demonstrate that the absence of OTA must be
435 due to the dominating effect of the co-inoculated yeasts over the OTA-producing moulds
436 (Photograph 2.2).
437 Despite the antagonistic effect observed in the speck model system at all of the Aw levels
438 tested, it is reasonable to propose that both yeasts should be inoculated after the smoking
439 phase and before the drying and seasoning steps, when the Aw is 0.95 ± 0.01 and the

moulds have not started to grow. The suggested concentration of the inoculum should be 10_6 CFU/cm_2 to absolutely ensure that the inoculum can predominate over the moulds. Different researchers (Rodriguez et al., 1994; Andrade et al., 2014) have suggested that yeast starter should be inoculated at the beginning of the production process for dry cured ham (specifically at the end of the post-salting stage), when the Aw of the product is still high (0.94) and can support OTA-producing mould growth. In our study, both of the inoculated strains grew rapidly on the FMCs at this Aw value and blocked the growth of OTA-producing moulds. Therefore, the biopreservative effect of the tested *D. hansenii* and *S. fibuligera* strains was obvious. Consequently, both strains may be proposed as antagonistic agents to prevent the presence of OTA-producing moulds and the bioaccumulation of OTA during speck production.

The complete prevention of OTA in speck can be obtained by the application of yeast co-inocula and of an adequate hygienic system based on Hazard Analysis and Critical Control Points (HACCP). The HACCP system can reduce the level of OTA-producing mould contamination on FMCs and speck and favour the antagonistic effect of both yeasts.

The sensorial acceptability of the speck inoculated with the starter cultures was confirmed by a triangular test using a panel composed of 20 non-professional assessors. These assessors did not find any difference between Lots A and B (with bioprotective cultures) and Lot C (uninoculated control).

In conclusion, *D. hansenii* and *S. fibuligera* are potential biopreservative agents for elimination of the growth of ochratoxigenic moulds in speck, a typical meat product of North Italy and Austria. The use of selected *D. hansenii* and *S. fibuligera* starter cultures, the control of raw meat and the technological (temperature and R.H.) and hygienic parameters are fundamental for the reduction of health hazards due to the development of OTA-producing moulds in dry-cured meat products, such as speck. Consequently, the

inoculation of *D. hansenii* or *S. fibuligera* strains after the smoking stage and during the drying and seasoning phases could improve the safety and quality of speck.

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Conflict of interest

None of the authors of this paper has a financial or personal relationship with other people or organisations that could inappropriately influence or bias the content of the paper.

Table 1: Flowchart of speck production

Production phases	Time	Temperature	R.H. %
Raw meat - Trimming	24 h	1-7 °C	50-60
Brining	8 day	1-5 °C	75-88
Smoking	170 h	15-22 °C	70-80
Drying	1 month	10-20 °C	50-90
Seasoning	5 months	15-20 °C	50-90

Legend: R.H.: Relative Humidity.

Table 2: Inhibitory activity of *Saccharomycopsis fibuligera* vs OTA producing moulds co-inoculated in agar plate.

Starter yeast strain	% Inibitory activity on	
	A. ochraceus	***P. nordicum***
S. fibuligera DIAL 1	70.6 ± 0.7a	71.0 ± 0.3a
S. fibuligera DIAL 2	75.5 ± 0.7b	75.4 ± 0.2b
S. fibuligera DIAL 3	86.1 ± 0.3c	85.2 ± 0.7c
S. fibuligera DIAL 4	69.1 ± 0.6d	68.5 ± 0.7d
S. fibuligera DIAL 5	80.2 ± 0.1e	78.5 ± 0.2e
S. fibuligera DIAL 6	76.5 ± 0.3b	75.2 ± 0.2 b

Legend: Data represent the means ± standard deviations of the total samples; Mean with the same letters within the same column (following the values) are not significantly differently ($P < 0.05$).

Table 3: Percentage of inhibitory activity of *Debaryomyces hansenii* vs OTA producing moulds co-inoculated in agar plates.

Starter yeast strain	% Inibitory activity on	
	A. ochraceus	***P. nordicum***
D. hansenii DIAL 1	76.0 ± 0.7a	78.8 ± 0.5a
D. hansenii DIAL 2	70.2 ± 0.3b	73.0 ± 0.7b
D. hansenii DIAL 3	70.3 ± 0.5b	72.2 ± 0.5b
D. hansenii DIAL 4	69.3 ± 0.7b	72.2 ± 0.3b
D. hansenii DIAL 5	68.0 ± 0.3c	72.0 ± 0.4b
D. hansenii DIAL 6	72.3 ± 0.5d	73.0 ± 0.5b

Legend: Data represent the means ± standard deviations of the total samples; Mean with the same letters within the same column (following the values) are not significantly differently ($p < 0.05$).

747 **Table 4: Percentage of inhibitory effect of different concentration of yeasts vs OTA**
748 **producing moulds co-inoculated in agar plates**
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Starter yeast strain	% Inibitory activity on	
	A. ochraceus	*P. nordicum*
***D. hansenii* DIAL 1**		
10^2 CFU/mL	38.4 ± 0.7a	31.0 ± 0.2a
10^4 CFU/mL	53.3 ± 0.5b	50.4 ± 0.3b
10^6 CFU/mL	76.0 ± 0.7c	78.8 ± 0.5c
***S. fibuligera* DIAL 3**		
10^2 CFU/mL	26.4 ± 0.2a	26.3 ± 0.5a
10^4 CFU/mL	54.2 ± 0.3b	51.1 ± 0.5b
10^6 CFU/mL	86.1 ± 0.3c	85.2 ± 0.7c

Legend: Data represent the means ± standard deviations of the total samples; Mean with the same letters within the same column (following the values) are not significantly differently ($p < 0.05$). *A. ochraceus* and *P. nordicum* concentration: 10_6 conidia/mL.

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Table 5: Percentage of inhibitory effect of yeasts vs different concentration of OTA producing moulds co-inoculated in agar plates

Starter yeast strain	% Inibitory activity on	
	D. hansenii	***S. fibuligera***
P. nordicum		
10^2 CFU/mL	76.2 ± 0.3a	79.3 ± 0.1a
10^4 CFU/mL	55.4 ± 0.4b	59.0 ± 0.2b
10^6 CFU/mL	38.5 ± 0.3c	42.5 ± 0.9c
A. ochraceus		
10^2 CFU/mL	78.5 ± 0.5a	85.0 ± 0.6a
10^4 CFU/mL	60.3 ± 0.4b	65.6 ± 0.2b
10^6 CFU/mL	42.0 ± 0.3c	48.0 ± 0.2c

Legend: Data represent the means ± standard deviations of the total samples; Mean with the same letters within the same column (following the values) are not significantly differently ($p < 0.05$). *D. hansenii* and *S. fibuligera* concentration: 10_4 CFU/mL.

Table 6: Mean production of Ochratoxin A by *P. nordicum* and *A. ochraceus* in control and selected yeast co-inoculated in speck model system with different Aw

	Ochratoxin A (µg/kg) in Speck					
Aw	***P. nordicum* Control**	***P. nordicum/ S. fibuligera***	***P. nordicum/ D. hansenii***	***A. ochraceus* Control**	***A. ochraceus/ S. fibuligera***	***A. ochraceus/ D. hansenii***
0.96	7.7	0.4	0.3	8.9	0.7	0.7
0.94	5.6	0.3	0.2	6.4	< 0.1	< 0.1
0.92	3.8	0.2	0.2	3.9	0.7	0.3
0.90	2.9	0.7	0.3	2.5	0.4	0.3
0.88	1.9	0.2	0.3	2.0	0.5	0.3

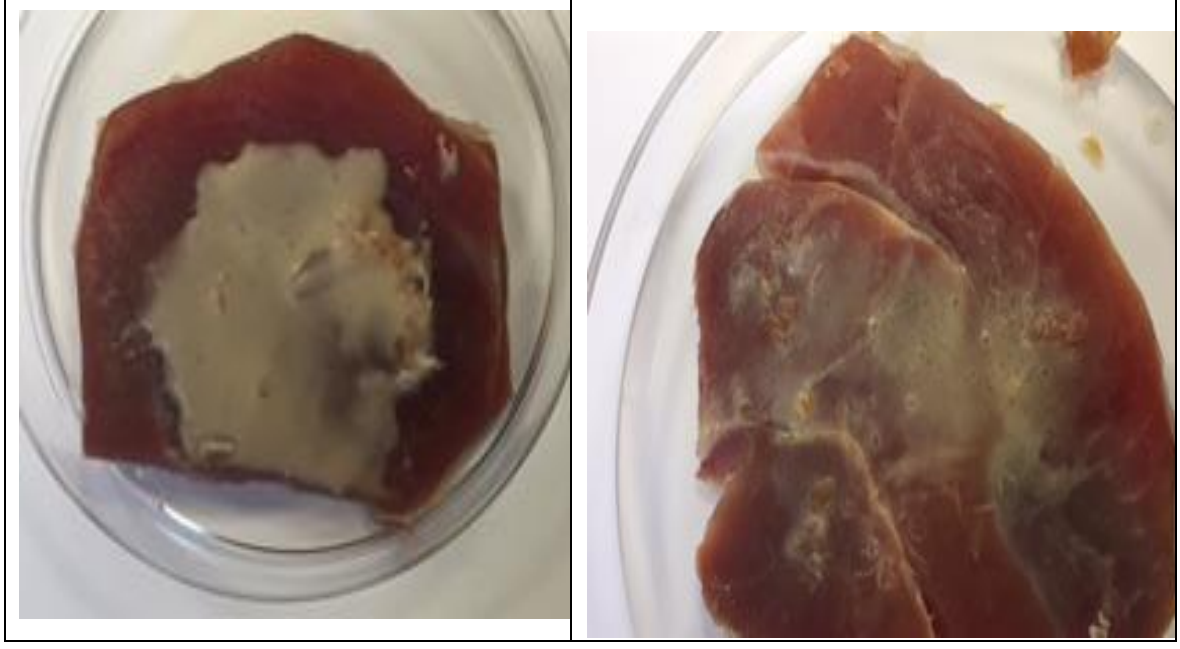
Legend: OTA Mean µg/kg.; LOD < 0.1 µg/kg; Samples were represented by meat pieces of 50 mm diameter and 5 mm in height.

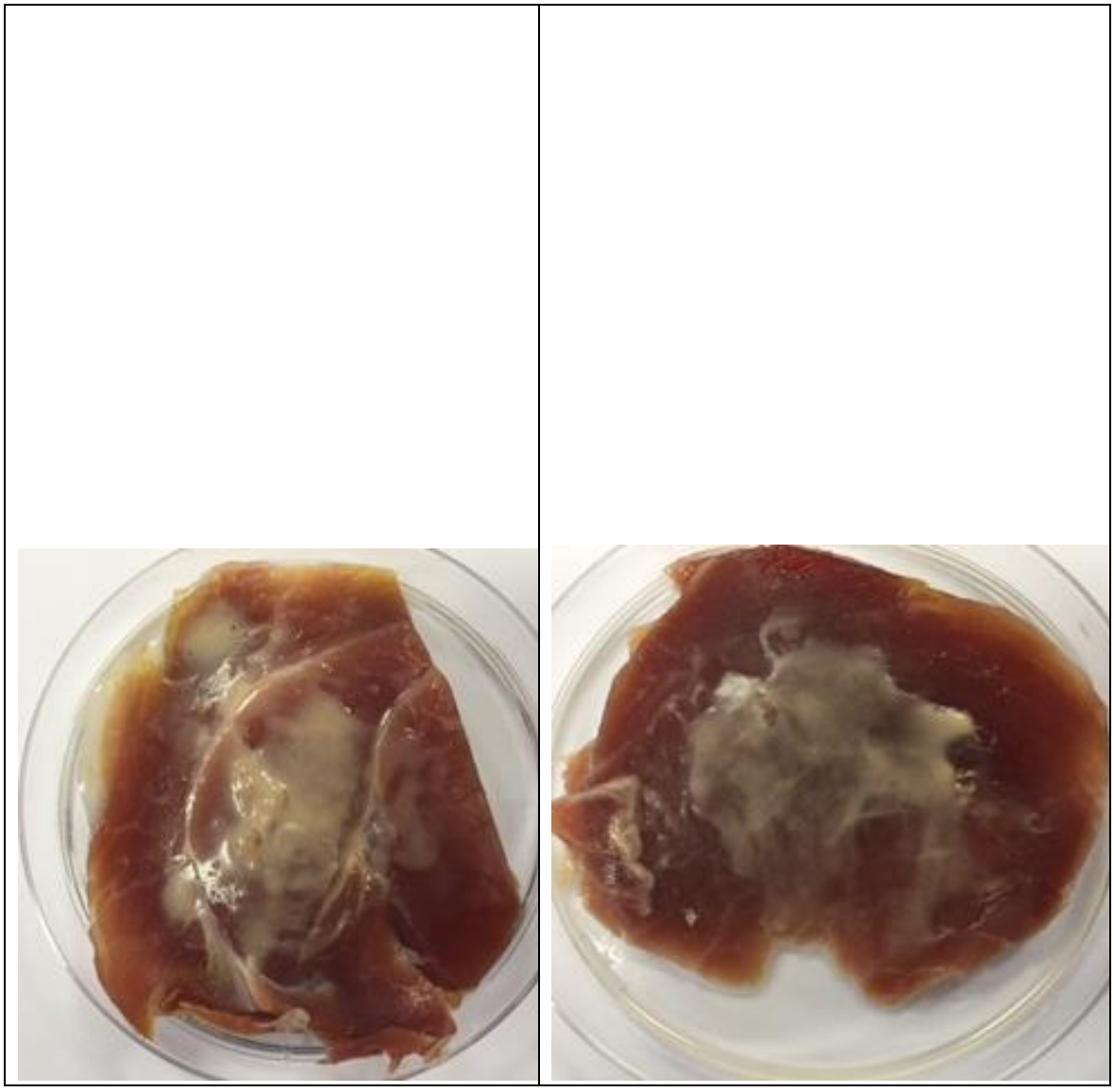
Table 7: Mean production of Ochratoxin A by *Penicillium nordicum* and *Aspergillus ochraceus* in control and selected yeast co-inoculated after smoking stage and valued during the seasoning of speck.

Toxigenic moulds	Ochratoxin A (µg/kg) in Speck			
	Speck inoculated with S. fibuligera	***Speck inoculated with D. hansenii***	**Control *A. ochraceus***	**Control *P. nordicum***
A. ochraceus	< 0.1	< 0.1	65.5 ± 1.5	-
P. nordicum	< 0.1	< 0.1	-	68.1 ± 1.9

Data: OTA mean ± standard deviations of 20 replicates: µg/kg.; LOD < 0.1 µg/kg; Samples taken from a depth of 0.5 cm below the surface; Inocolum after smoking.

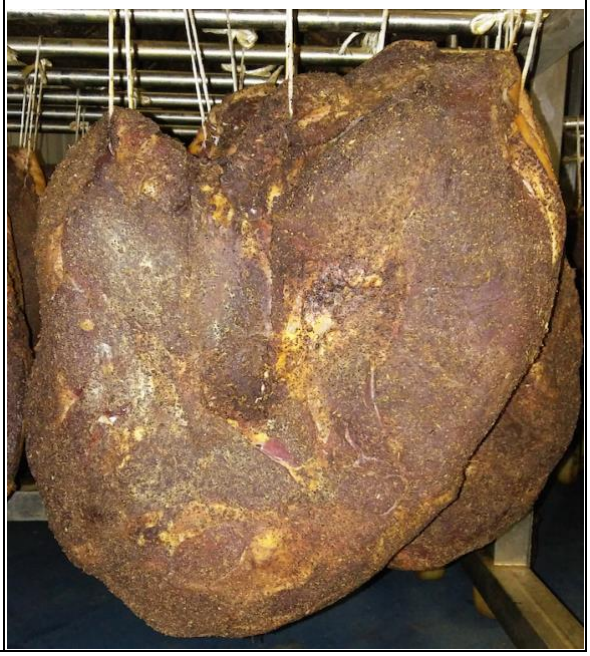
Photograph 1: Competition yeasts versus OTA producing moulds





Legend: 1-*D. hansenii vs P. nordicum*; 2-*E. fibuligera vs P. nordicum*
3-*D. hansenii* vs *A. ochraceus*; 4-*E. fibuligera* vs *A. ochraceus*

Photograph 2: Mould growth on speck without (1) and with yeast added (2)



Legend: 1 - *P. nordicum;* 2 - *D. hansenii* vs *P. nordicum*

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900 Photograph 1: Competition yeasts versus OTA producing moulds
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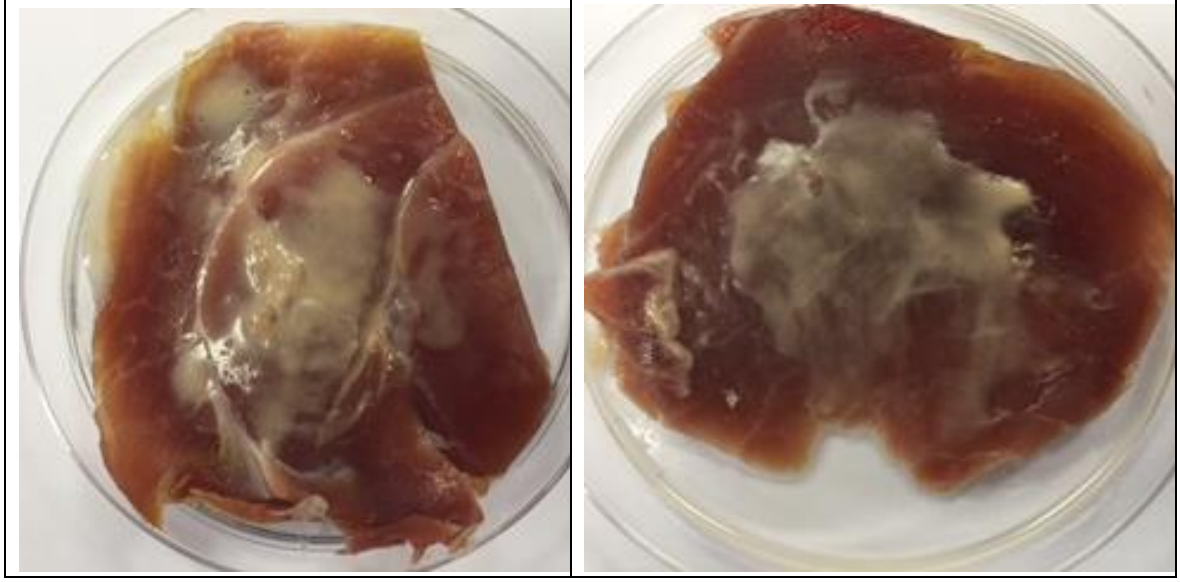
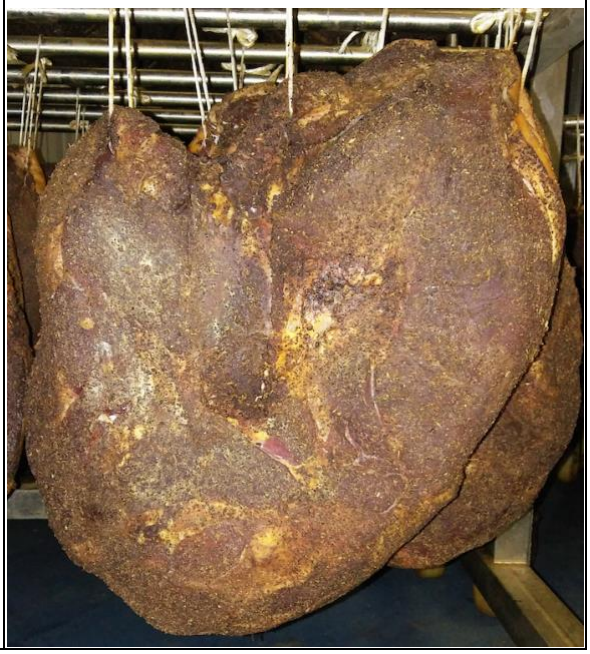


Legend: 1-*D. hansenii vs P. nordicum*; 2-*E. fibuligera vs P. nordicum*
3-*D. hansenii* vs *A. ochraceus*; 4-*E. fibuligera* vs *A. ochraceus*

Photograph 2: Mould growth on speck without (1) and with yeast added (2)



Legend: 1 - *P. nordicum;* 2 - *D. hansenii* vs *P. nordicum*