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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**

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

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49 **1. Introduction**

50 Speck is a typical meat product in the European alpine area, particularly North Tyrol  
51 (Austria) and South Tyrol (Alto Adige, Italy), regions that utilize different recipes but the  
52 same technology. A flow chart showing the traditional speck production process is  
53 presented in Table 1. Fresh meat cuts (FMC) obtained from deboned legs are carefully  
54 brined with a mixture of salt and spices, such as pepper, juniper, laurel and rosemary, and  
55 the percentages of these ingredients depend on the facility. The meat cuts are then cold  
56 smoked (20 °C), dehydrated and seasoned for four to six months at 10-15 °C with a  
57 relative humidity (R.H.) between 60 and 90%. During this period, moulds grow on the  
58 meat surface and give it a distinctive flavour. The colonization of speck during drying and  
59 seasoning occurs spontaneously from the mycobiota of the production environment or by  
60 contamination with spices (Peintner et al., 2000). The indigenous mycobiota are valued for  
61 their beneficial seasoning effects (Peintner et al., 2000). Moulds prevent excessive drying  
62 of the surface, improve the texture, and limit excessive hardness of the flesh due to their  
63 proteolytic activity (Sunesen and Stahnke, 2003). They also exerts an antioxidant effect,  
64 contribute to colour production (Spotti et al., 2008) and improve the aroma and flavour of  
65 speck (Rojas et al., 1991, Nunez et al., 1996). The surface moulds of meat products have  
66 been studied in great detail (Dragoni et al., 1980; Huerta et al., 1987; Spotti et al., 1989;  
67 Rojas et al., 1991; Nunez et al., 1996; Battilani et al., 2007; Iacumin et al., 2011). *Eurotium*  
68 spp. appears to be the most prevalent during all stages of meat production (Peintner et al.,  
69 2000; Comi et al., 2004; Battilani et al., 2010; Comi and Iacumin, 2013). In North and  
70 South Tyrol, *Eurotium rubrum* and *Penicillium solitum* are the dominant species in all  
71 parts of speck (crust, meat and fat). Other Pennicillia, including *P. nalgiovense*, *P.*  
72 *verrucosum*, *P. canescens*, *P. brevicompactum*, *P. chrysogenum*, *P. glabrum*, *P. commune*  
73 and *P. waksmanii*, are less frequently observed (Peintner et al., 2000). The development of  
74 mould can also be related to negative effects, such as the production of mycotoxin (Comi  
75 and Iacumin, 2013). Several recent investigations have highlighted the presence of

76 ochratoxin A (OTA) in different meat products, and several fungi, including *Penicillium*  
77 *verrucosum*, *P. nordicum*, and *Aspergillus ochraceus*, can produce OTA following growth  
78 on the surface of meat products during seasoning and storage (Matrella et al. 2006; Pietri et  
79 al. 2006; Iacumin et al., 2011). OTA is undesirable because it is classified by IARC  
80 (International Agency for Research of Cancer) into "Group B" as a molecule with possible  
81 carcinogenic activity in humans (IARC, 1993). For this reason, limits have been introduced  
82 to foods. In Italy, the maximum concentration of OTA allowed in meat and meat products  
83 is 1 µg/kg (Ministry of Health Circular no. 10-93 09/06/1999). Different artisanal  
84 production lots of speck were recently contaminated with *A. ochraceus* and *P. nordicum*.  
85 Both species grow during drying and seasoning and produce up to 1 µg/kg OTA. Many  
86 technologies must be used to eliminate the growth of OTA-producing moulds on meat  
87 products. The most interesting methods developed to date are represented by bioprotective  
88 cultures. Exploring the antagonistic activity of autochthonous yeast strains against moulds  
89 could be of great interest. Several researchers (Andrade et al., 2014; Virgili et al., 2012)  
90 have experimented with the ability of native yeasts to protect meat products against mould  
91 growth. *D. hansenii* is the main yeast present in meat products from the early stage of  
92 seasoning to the end, when it becomes the predominant strain (Comi and Cantoni, 1980;  
93 Nunez et al., 1996; Asefa et al., 2010; Simoncini et al., 2007). Consequently, *D. hansenii* is  
94 largely used as a starter culture to improve the quality and sensorial characteristics of  
95 European sausages (Hammes and Knauf, 1994). Conversely, *S. fibuligera* is rarely present  
96 in meat products because it is typical of starchy substrates and vegetables (Chi et al.,  
97 2009). However, it is used because it grows in the presence of high salt and sugar  
98 concentrations similarly to *D. hansenii*. Because yeasts are capable of growing and  
99 colonizing meat products, they could represent the main strains for food biocontrol against  
100 moulds and pathogenic microorganisms (Virgili et al., 2012; Andrade et al., 2014;  
101 Simoncini et al., 2014). The efficacy of the protection provided by yeasts against moulds

102 on meat products must be seriously evaluated because moulds often grow on meat product  
103 surfaces despite the presence of yeasts (Virgili et al., 2012). Little information regarding  
104 the antagonistic activity of yeasts on dry cured ham has been reported (Virgili et al., 2012),  
105 and different researchers have recently investigated and demonstrated the inhibitory effect  
106 of yeasts *in vitro* against OTA-producing moulds (Andrade et al., 2014; Simoncini et al.,  
107 2014). These researchers have demonstrated that it is possible to reduce *P. nordicum*  
108 growth and activity using different yeast strains. The inhibitory mechanism is not well  
109 understood, but it has been suggested that yeasts restrict nutrient availability and sites for  
110 colonization and consequently predominate and limit the growth of OTA-producing  
111 moulds (Bjönberg and Schnürer, 1993; Spotti et al., 2009). Considering the many  
112 opportunities that yeasts can have in mould inhibition, this study aimed to evaluate the  
113 abilities of *Debaryomyces hansenii* and *Saccharomycopsis fibuligera* to control  
114 ochratoxigenic mould growth (*Aspergillus ochraceus* and *Penicillium nordicum*) during  
115 speck production.

116

## 117 **2. Materials and methods**

118 Six strains of *Saccharomycopsis fibuligera* (DIAL 1, DIAL 2, DIAL 3, DIAL 4, DIAL 5,  
119 and DIAL 6) and six strains of *Debaryomyces hansenii* (DIAL 1, DIAL 2, DIAL 3, DIAL  
120 4, DIAL 5, and DIAL 6) were selected from a total of 90 isolates from meat products and  
121 stored in the Collection of the Department of Food Science of the University of Udine  
122 (DIAL, Udine, Italy). The twelve yeast strains were selected for potential competition  
123 against *Penicillium nordicum* and *Aspergillus ochraceus*. The strains were maintained in  
124 malt extract modified agar (MEMA, Oxoid, Italy) supplemented with dextrose (1%) and  
125 peptone (1%).

126

### 127 *2.1. Preparation of yeast inocula*

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

133

### 134 2.2. Preparation of the *P. nordicum* and *A. ochraceus* inocula

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

143

### 144 2.3. Inhibition of *P. nordicum* and *A. ochraceus* by yeast strains

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

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

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

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

#### 162 2.4. Inhibitory activity at different concentrations of the selected yeasts

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

173

#### 174 2.5. Inhibitory activity at different concentrations of moulds (*P. nordicum* and *A.* 175 *ochraceus*)

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



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

185

186 *2.6. Inhibitory activity of yeasts on OTA production in the speck model system* (Battilani et  
187 al., 2010, modified)

188 Five specks with different water activity ( $A_w$ ) values were collected from a South  
189 Tyrolean facility. From each speck, samples (50 mm in diameter and 5 mm in height) were  
190 excised using a hollow metal sampler with a cylindrical cutting edge. The  $A_w$  values of the  
191 samples measured with an AquaLab CX-2 instrument (Steroglass, Pullman, WA, USA)  
192 were  $0.960 \pm 0.005$ ,  $0.940 \pm 0.005$ ,  $0.920 \pm 0.005$ ,  $0.900 \pm 0.005$ , and  $0.880 \pm 0.005$ . After  
193 dipping in absolute ethanol for 2 min, the samples were removed and flamed to sterilize  
194 them prior to inoculation (Rojas et al., 1991). The samples were placed in Petri plates,  
195 inoculated and placed in sealed boxes equipped with beakers containing NaCl solutions  
196 (Multon and Bizot, 1980) with the same  $A_w$  as the five speck samples. The salt solutions  
197 were prepared with distilled water (w/w) with 6.57% NaCl ( $A_w$  0.96), 9.38% NaCl ( $A_w$   
198 0.94), 11.90% NaCl ( $A_w$  0.92), 14.18% NaCl ( $A_w$  0.90) and 16.28% NaCl ( $A_w$  0.88).  
199 Each  $A_w$  value was confirmed using AquaLab CX-2.

200 The samples were incubated in the dark at 14 °C for 30 days. Six different inocula or co-  
201 inocula were tested: 1) Control A,  $10^4$  conidia/cm $^2$  *Aspergillus ochraceus*; 2) Control B,  
202  $10^4$  conidia/cm $^2$  *Penicillium nordicum*; 3)  $10^6$  CFU/g *D. hansenii* DIAL 1 vs.  $10^4$   
203 conidia/cm $^2$  *Aspergillus ochraceus*; 4)  $10^6$  CFU/g *D. hansenii* DIAL 1 vs.  $10^4$  conidia/cm $^2$   
204 *Penicillium nordicum*; 5)  $10^6$  CFU/g *S. fibuligera* DIAL 3 vs.  $10^4$  conidia/cm $^2$  *Aspergillus*  
205 *ochraceus*; and 6)  $10^6$  CFU/g *S. fibuligera* DIAL 3 vs.  $10^4$  conidia/cm $^2$  *P. nordicum*. Each

206 condition was evaluated in triplicate. At the end of the incubation period, the 90 samples  
207 were collected and analysed for OTA according to the method reported by Matrella et al.  
208 (2006).

209

### 210 *2.7. Inhibitory activity of yeasts on OTA production during speck seasoning*

211 One hundred and twenty fresh meat cuts (FMCs) were collected. Each FMC (6.5 kg) was  
212 trimmed and squared according to the traditional procedure and was then salted and  
213 smoked. After smoking, the FMCs were inoculated with a mix of yeast and mould  
214 suspensions or with a mould suspension alone according to the following experimental  
215 design: 20 FMCs were inoculated with a suspension of *P. nordicum* at a final concentration  
216  $10^4$  conidia/cm<sup>2</sup>; 20 FMCs were inoculated with a suspension of *A. ochraceus* at a final  
217 concentration  $10^4$  conidia/cm<sup>2</sup>; 20 FMCs were inoculated with a mix of *A. ochraceus* and  
218 *D. hansenii* (final concentration  $10^4$  conidia/ $10^6$  CFU/cm<sup>2</sup>); 20 FMCs were inoculated with  
219 a mix of *A. ochraceus* and *S. fibuligera* ( $10^4$  conidia/ $10^6$  CFU/cm<sup>2</sup>); 20 FMCs were  
220 inoculated with a mix of *P. nordicum* and *D. hansenii* ( $10^4$  conidia/ $10^6$  CFU/cm<sup>2</sup>); and 20  
221 FMCs were inoculated with a mix of *P. nordicum* and *S. fibuligera* ( $10^4$  conidia/ $10^6$   
222 CFU/cm<sup>2</sup>). The *S. fibuligera* DIAL 3 and *D. hansenii* DIAL 1 strains were used. All of the  
223 FMCs were seasoned for six months according to the traditional procedure (Table 1). At  
224 the end of the seasoning period, 100-cm<sup>2</sup> samples were taken from a depth of 0.5 cm below  
225 the speck surface and analysed for OTA. Briefly, meat collected from a depth of 0.5 cm  
226 below the slime was homogenized using a Stomacher instrument (Lab Blender 400, PBI,  
227 Italy), and 10 g of the homogenate was then used for the analysis. OTA was extracted and  
228 evaluated according to the method described by Matrella et al. (2006). Before sampling for  
229 OTA analysis, the surface of each speck was observed with a Stereoscope (320X; WILD  
230 M 420, Heerbrugg, CH) to determine the presence of mould growth (i.e., hyphae).

231

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<sup>2</sup>), and Lot B (10 cuts)  
237 was inoculated with *D. hansenii* DIAL 1 ( $10^6$  CFU/cm<sup>2</sup>). 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).

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

265 For this reason, both strains were selected for the assessment of their *in vitro* or *in vivo*  
266 inhibitory effects. Different concentrations of both yeast strains were tested separately to  
267 identify the best strain. As expected, the inhibitory activity was dependent on the yeast  
268 concentration. Indeed, an *in vitro* concentration of  $10^6$  CFU/mL of agar showed the best  
269 inhibitory effect for both of the tested yeasts ( $p < 0.05$ ; Table 4). The antagonistic activities  
270 of the yeasts ( $10^4$  CFU/mL) were evaluated separately against different concentrations of  
271 both moulds ( $10^2$ ,  $10^4$ , and  $10^6$  conidia/mL). The percentage of inhibition varied according  
272 to the mould concentration (Table 5). The lowest percentage of inhibition was observed  
273 after inoculation with the highest mould concentration ( $p < 0.05$ ). The activity of *S.*  
274 *fibuligera* was higher than the activity of *D. hansenii*. A higher yeast concentration (i.e.,  
275  $10^6$  CFU/mL) completely inhibited mould at concentrations of  $10^2$  and  $10^4$  conidia/mL  
276 (data not shown), and the same results, which are shown in Table 4, were obtained with  
277 similar yeast and mould concentrations ( $10^6/10^6$  CFU/mL). The measured diameters  
278 demonstrated that the inhibitory activity of *S. fibuligera* was higher than that of *D. hansenii*  
279 ( $p < 0.05$ ), even though the media used appeared to be more productive for *D. hansenii*  
280 than for *S. fibuligera*.

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

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  $A_w$  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

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

314

#### 315 **4. Discussion**

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

321 Speck is a typical meat product of North Tyrol (Austria) and South Tyrol (Alto Adige,  
322 Italy). In Italy, speck is also produced in the Veneto and Friuli Venezia Giulia regions.  
323 During the seasoning process, a large population of microorganisms (mainly moulds and  
324 yeasts) develops on the outer layers of the speck (Peintner et al., 2000). Some of these  
325 moulds could produce OTA, such as *P. nordicum* and *A. ochraceus*. Under some  
326 environmental conditions, their growth and ability to synthesize OTA could represent a  
327 real risk for human health. OTA has been found in different meats and meat products  
328 contaminated by ochratoxigenic moulds (Rojas et al., 1991; Battilani et al., 2010;  
329 Dall'Asta et al., 2010; Iacumin et al., 2011). However, the presence of OTA is not always  
330 correlated with OTA-producing moulds in meat products (Rodríguez et al., 2012).  
331 Different methods can be used to inhibit OTA-producing moulds (Iacumin et al., 2011;  
332 Comi et al., 2013). The most commonly used method consists of spreading starter cultures  
333 on the meat product surface in the late drying and seasoning stages (Comi et al., 2013). In  
334 sausage production, mould and yeast starters are mainly used because they limit the growth  
335 of pathogenic microorganisms and because they improve the sensorial quality of the

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

342 The inhibitory effect of *S. fibuligera* DIAL 3 against both of the mould strains was higher  
343 and significantly different ( $p < 0.05$ ) compared with the effect of *D. hansenii* DIAL 1. The  
344 inhibitory activity of *D. hansenii* strains against *P. nordicum* was also found to be reduced  
345 by Virgili et al. (2012), which is in agreement with the results of our investigation. These  
346 researchers found that two strains, which were selected for their ability to grow in dry  
347 cured ham-like substrates (*Candida zeylanoides* and *Hyphopichia burtonii*), were more  
348 effective against *P. nordicum* than against *D. hansenii* and *Candida famata* (the  
349 anamorphic form of *D. hansenii*). Indeed, both of these strains inhibited *P. nordicum*  
350 growth and OTA production (Virgili et al., 2012). Differences in inhibitory activity against  
351 *P. nordicum* were also found among *D. hansenii* strains among Andrade et al. (2014).  
352 These researchers demonstrated that the *in vitro* efficiency depends on the strains, the  
353 medium and the  $A_w$  value and that the lysed yeast cells might provide nutrients for *P.*  
354 *nordicum* growth. These researchers concluded that the inhibition observed appears to be  
355 due to compounds produced by yeast strains with activity against the tested moulds.  
356 Several researchers (Marquina et al., 2001; Masoud et al., 2005) have demonstrated that *D.*  
357 *hansenii*, *Saccharomyces cerevisiae* and *Pichia anomala* produce killer proteins against  
358 sensitive strains of yeasts and moulds. Again, the observed mould inhibition could be due  
359 to the competition activity of nutrients, as previously suggested (Björnberg and Schnürer,  
360 1993; Zhao et al., 2008); however, researchers have speculated the existence of a minor  
361 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  $A_w$ , the yeasts or the mould strains. The reduced OTA concentration in the speck model  
381 system with different  $A_w$  values compared with the control samples was quite obvious.  
382 The antagonistic activities of *D. hansenii* and *S. fibuligera* were not dependent on the  $A_w$   
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 ( $>1 \mu\text{g}/\text{kg}$ ) 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 ( $1 \mu\text{g}/\text{kg}$ ). 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 ( $< 0.1 \mu\text{g}/\text{kg}$ ). 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  $0.95 \pm 0.01$  and the

440 moulds have not started to grow. The suggested concentration of the inoculum should be  
441  $10^6$  CFU/cm<sup>2</sup> to absolutely ensure that the inoculum can predominate over the moulds.  
442 Different researchers (Rodriguez et al., 1994; Andrade et al., 2014) have suggested that  
443 yeast starter should be inoculated at the beginning of the production process for dry cured  
444 ham (specifically at the end of the post-salting stage), when the *A<sub>w</sub>* of the product is still  
445 high (0.94) and can support OTA-producing mould growth. In our study, both of the  
446 inoculated strains grew rapidly on the FMCs at this *A<sub>w</sub>* value and blocked the growth of  
447 OTA-producing moulds. Therefore, the biopreservative effect of the tested *D. hansenii* and  
448 *S. fibuligera* strains was obvious. Consequently, both strains may be proposed as  
449 antagonistic agents to prevent the presence of OTA-producing moulds and the  
450 bioaccumulation of OTA during speck production.

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

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

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

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

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602

603 **Conflict of interest**

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

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**Table 1: Flowchart of speck production**

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

Legend: R.H.: Relative Humidity.

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

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

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).

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

Starter yeast strain	% Inibitory activity on	
	<i>A. ochraceus</i>	<i>P. nordicum</i>
<i>D. hansenii</i> DIAL 1	76.0 ± 0.7a	78.8 ± 0.5a
<i>D. hansenii</i> DIAL 2	70.2 ± 0.3b	73.0 ± 0.7b
<i>D. hansenii</i> DIAL 3	70.3 ± 0.5b	72.2 ± 0.5b
<i>D. hansenii</i> DIAL 4	69.3 ± 0.7b	72.2 ± 0.3b
<i>D. hansenii</i> DIAL 5	68.0 ± 0.3c	72.0 ± 0.4b
<i>D. hansenii</i> 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 ( $p < 0.05$ ).

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**Table 4: Percentage of inhibitory effect of different concentration of yeasts vs OTA producing moulds co-inoculated in agar plates**

Starter yeast strain	% Inibitory activity on	
	<i>A. ochraceus</i>	<i>P. nordicum</i>
<b><i>D. hansenii</i> DIAL 1</b>		
10 <sup>2</sup> CFU/mL	38.4 ± 0.7a	31.0 ± 0.2a
10 <sup>4</sup> CFU/mL	53.3 ± 0.5b	50.4 ± 0.3b
10 <sup>6</sup> CFU/mL	76.0 ± 0.7c	78.8 ± 0.5c
<b><i>S. fibuligera</i> DIAL 3</b>		
10 <sup>2</sup> CFU/mL	26.4 ± 0.2a	26.3 ± 0.5a
10 <sup>4</sup> CFU/mL	54.2 ± 0.3b	51.1 ± 0.5b
10 <sup>6</sup> 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<sup>6</sup> 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	
	<i>D. hansenii</i>	<i>S. fibuligera</i>
<b><i>P. nordicum</i></b>		
10 <sup>2</sup> CFU/mL	76.2 ± 0.3a	79.3 ± 0.1a
10 <sup>4</sup> CFU/mL	55.4 ± 0.4b	59.0 ± 0.2b
10 <sup>6</sup> CFU/mL	38.5 ± 0.3c	42.5 ± 0.9c
<b><i>A. ochraceus</i></b>		
10 <sup>2</sup> CFU/mL	78.5 ± 0.5a	85.0 ± 0.6a
10 <sup>4</sup> CFU/mL	60.3 ± 0.4b	65.6 ± 0.2b
10 <sup>6</sup> 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<sup>4</sup> CFU/mL.

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**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 ( $\mu\text{g}/\text{kg}$ ) in Speck						
Aw	<i>P. nordicum</i> Control	<i>P. nordicum</i> / <i>S. fibuligera</i>	<i>P. nordicum</i> / <i>D. hansenii</i>	<i>A.</i> <i>ochraceus</i> Control	<i>A.</i> <i>ochraceus</i> / <i>S. fibuligera</i>	<i>A.</i> <i>ochraceus</i> / <i>D. hansenii</i>
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  $\mu\text{g}/\text{kg}$ .; LOD < 0.1  $\mu\text{g}/\text{kg}$ ; Samples were represented by meat pieces of 50 mm diameter and 5 mm in height.

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**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.**

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Toxigenic moulds	Ochratoxin A (µg/kg) in Speck			
	<i>Speck inoculated with S. fibuligera</i>	<i>Speck inoculated with D. hansenii</i>	Control <i>A. ochraceus</i>	Control <i>P. nordicum</i>
<i>A. ochraceus</i>	< 0.1	< 0.1	65.5 ± 1.5	-
<i>P. nordicum</i>	< 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; Inoculum after smoking.

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Photograph 1: Competition yeasts versus OTA producing moulds





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862 Legend: 1-*D. hansenii* vs *P. nordicum*; 2-*E. fibuligera* vs *P. nordicum*  
863 3-*D. hansenii* vs *A. ochraceus*; 4-*E. fibuligera* vs *A. ochraceus*

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878 Photograph 2: Mould growth on speck without (1) and with yeast added (2)  
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Legend: 1 - *P. nordicum*; 2 - *D. hansenii* vs *P. nordicum*

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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*

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



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