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Biocontrol of *Penicillium griseofulvum* to reduce cyclopiazonic acid contamination in dry-fermented sausages

Josué Delgado[1], Belén Peromingo, Alicia Rodríguez, Mar Rodríguez*

Food Hygiene and Safety, Meat and Meat Products Research Institute, Faculty of Veterinary Science, University of Extremadura, Avda, de las Ciencias, s/n, 10003 Cáceres, Spain

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ABSTRACT

Dry-fermented sausages are very appreciated by consumers. The environmental conditions during its ripening favor colonization of their surface by toxigenic molds. These molds contribute to the development of sensory characteristics; however, some of them could produce mycotoxins such as cyclopiazonic acid (CPA). CPA is mainly produced by *Penicillium commune* and *Penicillium griseofulvum* which have been found in dry-cured meat products. Thus, strategies to prevent the CPA contamination in dry-fermented sausages are needed. The objective of this work was to evaluate the ability of *P. griseofulvum* to produce CPA in dry-fermented sausage during its ripening as well as to test different strategies to prevent CPA production. The ability of PgAFP antifungal protein-producing *Penicillium chrysogenum*, *Debaryomyces hansenii* and *Pediococcus acidilactici* for inhibiting CPA production by *P. griseofulvum* was tested on dry-fermented sausage-based medium. Only *P. chrysogenum* inhibited the CPA production, so this mold was co-inoculated with *P. griseofulvum* on sausages whose ripening was performed at low temperature. CPA reached around 800 ng/g in the control batch, being reduced to 20 ng/g by the presence of *P. chrysogenum*. This work demonstrates the risk posed by CPA on dry-fermented sausages, and provides a successful strategy to prevent this hazard.

1. Introduction

Dry-fermented sausage is one of the most typical Spanish dry-cured meat product. This kind of product is produced in southern Europe, being highly appreciated by the consumers. The usual microbiota present in this product is comprised by *Lactobacilli* together with yeasts and molds, mainly belonging to *Debaryomyces hansenii* and *Penicillium* spp., respectively. These microorganisms contribute to the development of the required sensory characteristics of dry-fermented sausages (Cano-García et al., 2014; Fadda et al., 1999; Martín et al., 2006).

However, the environmental conditions during the ripening of dry-cured meat products also favor growth of toxigenic molds on their surface (López-Díaz et al., 2001; Núñez et al., 1996). In addition, some of these molds can produce mycotoxins on such meat products (Peromingo et al., 2018b; Pleadin et al., 2015; Rodríguez et al., 2012a, 2012b). Although ochratoxin A (OTA) is the most frequently encountered mycotoxin in dry-cured meat products, cyclopiazonic acid (CPA) has been also detected in salami (Ostry et al., 2018) and even in dry-cured ham (Alapont et al., 2014; Bailly et al., 2005; Peromingo et al., 2018b). The latter mycotoxin is produced by mold strains commonly found in dry fermented sausages and other meat products, such as *Penicillium commune* and *Penicillium griseofulvum* (Alapont et al., 2014; Bailly et al., 2005; Galvalisi et al., 2012; López-Díaz et al., 2001; Ostry et al., 2018). CPA may produce adverse effects in consumers since it is a specific inhibitor of calcium-dependent ATPase in the sarcoplasmic reticulum and it provokes weight loss, nausea, diarrhea, giddiness, muscle necrosis, convulsions, and neurochemical and mutagenic toxicity (Ostry et al., 2018). There is very little proof for human toxicity due to consumption of food contaminated with CPA (Ostry et al., 2018). In addition, CPA has been found to be stable in dry-cured meat (Bailly et al., 2005). Therefore, it is necessary to test whether the dry-fermented sausage ripening allows the CPA production in order to design effective strategies to prevent this mycotoxin contamination in this product.

The usual temperature during dry-fermented sausage ripening is around 12 °C and the relative humidity (RH) is higher than 75%

* Corresponding author.
E-mail address: marrodri@unex.es (M. Rodríguez).
URL: http://higiene.unex.es/ (M. Rodríguez).
[1] Present position: Heart Clinical Unit, Virgen de la Victoria University Clinic Hospital, Institute of Biomedical Research in Malaga IBIMA, CIBERCV, University of Málaga, Málaga, Spain.

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(Bernáldez et al., 2013). Under these conditions, it is necessary to test the ability of *P. griseofulvum* to grow and produce CPA. Among the different strategies to control unwanted molds, the biocontrol is the best accepted by the consumers, given that they demand residue-free chemical foods. However, it is of utmost importance to determine the capacity of the biocontrol agents to grow and compete against toxigenic molds at the ripening conditions of dry-fermented sausages.

Among these agents, some molds produce proteins that delays other molds growth, whereas the activity against yeasts and prokaryotes is quite limited (Delgado et al., 2015). The antifungal protein PgAFP, produced by *Penicillium chrysogenum* CECT 20922, inhibits some toxigenic molds in culture medium and on dry-fermented sausage. However, this effect is rather time-limited, displaying the main effect during the first 96 h of incubation at 25 °C (Delgado et al., 2015). For this reason, it would be interesting to test the ability of *P. chrysogenum* to control CPA producing molds by producing PgAFP or utilizing other mechanisms.

Yeasts have also shown to be effective for the control of ochratoxigenic molds on dry-fermented sausages (Andrade et al., 2014; Núñez et al., 2015). This inhibition has been explained by the production of volatile compounds as well as the nutrient and substrate competition. Besides lactic acid bacteria have been proved as potent mold growth inhibitors, *Pediococcus acidilactici* produces antimicrobial compounds, being effective against some *Penicillium* spp. (Mandal et al., 2013).

Therefore, the objective of this work was to evaluate the ability of *P. griseofulvum* to produce CPA in dry-fermented sausage during its ripening as well as to test different strategies to prevent this mycotoxin production.

2. Material and methods

2.1. Bacterial and fungal strains

The PgAFP antifungal protein-producing *P. chrysogenum* 20922, from the Spanish Type Culture Collection (CECT, Valencia, Spain), and *P. griseofulvum* 14319, from the Type Culture Collection of the Department of Biotechnology from the Technical University of Denmark (IBT, Lyngby, Denmark), were used in this study. In addition, two potential antifungal strains were used: *D. hansenii* Dh253 from the Culture Collection of Food Hygiene and Safety at the University of Extremadura (FHSCC, Cáceres, Spain) and *P. acidilactici* fargo 35 supplied by Laboratorios Amerex (Colmenar Viejo, Spain).

2.2. Inoculum preparation

P. chrysogenum and *P. griseofulvum* were grown on potato dextrose agar (Scharlau Chemie S.A., Spain) for 7 days at 25 °C in order to harvest conidia with sterile phosphate-saline buffer (PBS) at the end of the incubation time. *D. hansenii* was grown in yeast extract sucrose broth (YES) for 72 h at 200 rpm and 25 °C. YES medium containing *D. hansenii* was centrifuged to concentrate cells and they were washed twice with sterile PBS. *P. acidilactici* was grown in MRS broth (Man, Rogosa and Sharpe; Scharlau Chemie S.A.) for 24 h at 30 °C. After incubation time, the procedure followed by *D. hansenni* to concentrate the cells was also used. Spore or yeast suspensions were counted in a Thoma counting chamber Blaubrand® (Brand, Wertheim, Germany), and adjusted to the required concentration by diluting with PBS. Bacteria inoculation was performed by using data from previous assays to inoculate the desired concentration; the counts were checked by counting in MRS agar for 24 h at 30 °C.

2.3. PgAFP purification

The PgAFP producing *P. chrysogenum* CECT 20922 was inoculated into malt extract broth (20 g/L malt extract, 20 g/L glucose, and 1 g/L peptone), pH was adjusted at 4.5, and incubated up to 21 days at 25 °C. PgAFP was obtained from the cell free medium by fast protein liquid chromatography with a cationic exchange column HiTrap SP HP (Amersham Biosciences, Uppsala, Sweden), further purified with a HiLoad 26/60 Superdex 75 gel filtration column for FPLC (Amersham Biosciences), and concentrated as previously described (Acosta et al., 2009). PgAFP concentration in a pooled stock solution was measured by Lowry method (Lowry et al., 1951), sterilized through 0.22 µm acetate cellulose filters (Fisher Scientific, United Kingdom), and stored at −20 °C until use.

2.4. Experimental settings

2.4.1. Sensitivity to PgAFP

In order to test the *P. griseofulvum* sensitivity against PgAFP, a microplate with seven PgAFP concentrations (1.2–75 µg/mL) was prepared using potato dextrose broth as culture medium. Each well was inoculated with 10^5 conidia and was incubated at 25 °C for up to 96 h and measured at 595 nm. The assay was run in sextuplicate wells and repeated once.

2.4.2. Effect of protective cultures on growth and mycotoxin production by *P. griseofulvum*

To test the effect of biocontrol agents on growth and CPA production by *P. griseofulvum*, dry-fermented-sausage agar-based medium plates were prepared as described previously (Rodríguez et al., 2015), adding NaCl to reach the same level used for dry-fermented sausages processing (1.8%). The water activity (a_w) values were adjusted to 0.956 and 0.924 with 12.5 and 22% (v/v) of glycerol, respectively. These two a_w values were chosen to simulate the a_w values of dry-fermented sausages just after stuffing and in the middle of the ripening of this product. The media were poured into 58 mm diameter petri plates.

2.4.2.1. Interaction between P. griseofulvum and protective cultures. For this purpose, the methodology described previously (Magan and Lacey, 1984), was adapted to fermented-sausage agar-based medium plates. Plates were inoculated at two points separated by 38 mm with: a) 2 µL from a 10^7 spore suspension of *P. griseofulvum* and b) 2 µL from a 10^7 spore or cell suspension of *P. chrysogenum, D. hansenii* or *P. acidilactici*.

The experiments were done with two replicates and repeated once. Plates were incubated at 12 °C for up to 18 days. Pictures of the plates were taken pictures daily, from the day 11 to day 15 of incubation.

2.4.2.2. Protective cultures effect on fermented-sausage agar-based medium. To investigate the protective cultures effect on CPA production by *P. griseofulvum*, the dry-fermented sausage-based media adjusted at 0.956 a_w were inoculated by spreading 10^3 conidia or cells/cm^2 of protective culture or toxigenic mold. For this assay, three strains were used: *P. griseofulvum*, *P. chrysogenum* and *D. hansenii*. However, *P. acidilactici* was not used in this experiment due to its absence of growth in the conditions tested in Section 2.4.2.1. Four batches were prepared: batch Pg + Pc (inoculated with a mix of *P. griseofulvum* and *P. chrysogenum*), batch Pg + Dh (inoculated with a mix of *P. griseofulvum* and *D. hansenii*), batch Pg + Pc + Dh (inoculated with a mix of *P. griseofulvum*, *P. chrysogenum* and *D. hansenii*) and batch Pg (inoculated with *P. griseofulvum*). All the batches were incubated at 12 °C for 21 days, and the sampling for CPA analysis was carried out at the end of the incubation period. The experiment was made in triplicate and repeated once.

2.4.2.3. Protective cultures effect on dry-fermented sausage ripening. To test the potential of the biocontrol agent selected, commercial raw dry-fermented sausage of 40 mm of diameter (pH 5.8, 0.95 a_w) shortly after stuffing in artificial casing were ripened under usual conditions. The stuffing was made from minced Iberian pork meat (90%) and Iberian pig fatback (7%), with an addition of NaCl (1.8%), sugar, dextrose,

black pepper, and spices.

Sausages were inoculated by immersion in a spore solution of 10^6 conidia/mL from *P. griseofulvum* or *P. griseofulvum* + *P. chrysogenum* to reach *c.a.* 10^3 conidia/cm^2. The protective culture was chosen based on the results from the previous assay described in the Section 2.4.2.2. *P. chrysogenum* was the microorganism that showed the best results. Sausages were then incubated for 21 days: 3 days at 5 °C and 85% RH, 1 day at 13 °C and 84% RH and 18 days at 12 °C and 84% RH following the usual ripening process (Bernáldez et al., 2013) to reach *c.a.* 0.893 a_w. Sampling was done at days 14 and 17 for RNA extraction, and 14, 17 and 21 for CPA analysis. This experiment was prepared in triplicate and repeated once.

2.5. Gene expression studies related to the biosynthesis of protein PgAFP

2.5.1. RNA extraction

For RNA extraction, the mycelium was frozen in liquid nitrogen and ground with a sterile mortar and pestle, and resuspended in 750 µL RLT buffer (RNeasy® Plant Mini kit, Qiagen, Hilden, Germany) containing 15 µL β-mercaptoethanol. Samples were processed with the RNeasy® Plant Mini kit according to manufacturer's instructions. To remove genomic DNA contamination, samples were treated with DNase I, RNase-free (Fermentas, St. Leon-Rot, Germany) following manufacturer's instructions.

2.5.2. Reverse transcription real-time PCR assays and absolute quantification

Reverse transcription real-time PCR (RT-qPCR) based on SYBR Green methodology was performed to amplify the *PgAFP* gene from *P. chrysogenum* to unveil if the production of PgAFP is involved in the protective mechanism of this strain under the conditions tested. The primers F-Pc and R-Pc were previously optimized (Bernáldez et al., 2014).

2.5.2.1. cDNA synthesis. cDNA was synthesized using about 500 ng of total RNA according to the instructions of the PrimeScript™ RT Reagent kit protocol (Takara Bio Inc., Otsu, Shiga, Japan) and subsequently used for qPCR.

2.5.2.2. Quantification of the absolute expression of the PgAFP gene. Real-time PCR (qPCR) analyses were carried out in an Applied Biosystems ViiA TM 7 Real-Time PCR System (Applied Biosystems, USA) using the cDNA previously synthesized. qPCR reactions were essentially performed using the previously optimized conditions (Bernáldez et al., 2014) and the data analysis was carried out using the software by the ViiA™ 7 software version 1.2.4. (Applied Biosystems). Three replicates of samples together with a template-free negative control were also included in the runs.

The absolute quantification of the expression of the *PgAFP* gene was made by building standard curves using 10-fold DNA dilutions of *P. chrysogenum* strain ranging from 10 to 0.001 ng were prepared. The detection limits and amplification efficiencies were calculated. Quantification cycle (Cq), which is the intersection between each fluorescence curve and a threshold line, was automatically calculated by the instrument using default parameters. Absolute expression levels of the *PgAFP* gene were extrapolated from the standard curve by using the Cq values obtained for such samples.

2.6. Extraction and quantification of CPA

2.6.1. CPA extraction

For the extraction of CPA, methods 3 (for dry-fermented sausages) and 4 (dry-fermented sausage-agar based medium) based on QuEChERS procedure described by (Peromingo et al., 2018b) were used. Briefly, method 3 consisted in treating around 3 g of sample with 2 mL of water containing 0.1% (v/v) of acetic acid and mixed with a vortex for 30 s. Next, 2 mL of acetonitrile with acetic acid 0.1% (v/v) were added to the samples and they were vortexed for 1 min. In addition, 0.4 ± 0.01 g of sodium chloride and 1.6 ± 0.01 g of anhydrous magnesium sulfate were added to the samples before being vigorously shaken by hand for 15 s. Then the samples were centrifuged at 5000 rpm for 5 min at 25 °C and an aliquot of 1 mL of supernatant was transferred to 5 mL amber glass roller bottles. Method 4 consisted of a modification of method 3. In this method, all solvents and reagents used for CPA extraction were halved in relation to those used in methods 3 and the quantity of sample used was 1 g.

2.6.2. CPA analysis and quantification

The ultra-performance liquid chromatography (UHPLC) system used for CPA analyses was a Thermo Scientific Dionex UltiMate 3000 Rapid Separation LC (RSLC) system with an autosampler thermostat (UltiMate® 3000 Rapid Separation Autosampler, Thermo Scientific, Waltham, USA) coupled to an Ion Trap Mass Spectrometer System amaZon S.L. (Bruker Daltonics Inc., Bremen, Germany). The UHPLC-MS/MS method used was the previously described by Peromingo et al. (2018b). The limits of detection (LOD) and quantification (LOQ) were 3.3 and 10 ng/g, respectively, regardless of extraction method utilized.

2.7. Statistical analysis

Statistical analysis was performed using the SPSS v.24.0 software. Data sets of absorbance, *PgAFP* gene copy number and toxin production were tested for normality using the Shapiro-Wilk test. All data sets failed the normality test. Therefore, non-parametric data analysis was performed using the Kruskal-Wallis rank sum test. After that, the Mann-Whitney *U* test was applied to compare the median values obtained. The statistical significance was set at $p \leq 0.05$.

3. Results

3.1. Sensitivity to PgAFP

The study of the inhibition provoked by different concentrations of PgAFP revealed that from 48 h incubation, concentrations from 4.69 µg/mL PgAFP provoked a lower growth ($p \leq 0.05$) on *P. griseofulvum* (Fig. 1). This inhibition was observed from 13 to 24% for concentrations from 4.69 to 75 µg/mL respectively. According to absorbance data, the inhibition at 96 h incubation was higher than 40% from 9.38 µg/mL PgAFP and higher than 30% at 2.34 and 4.69 µg/mL PgAFP.

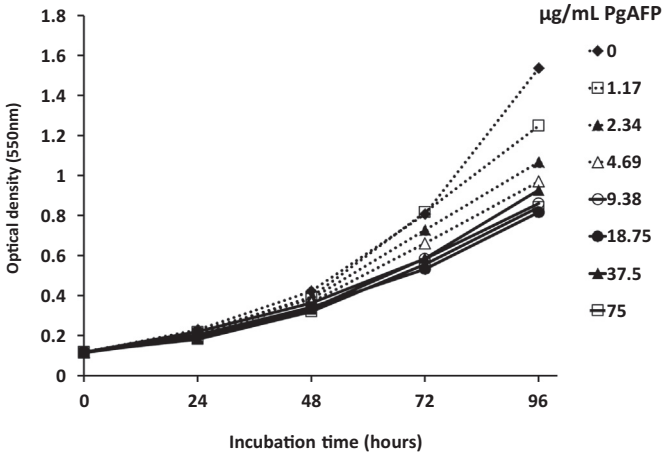

Fig. 1. Effect of different concentrations of PgAFP antifungal protein on *P. griseofulvum* growth for 96 h.

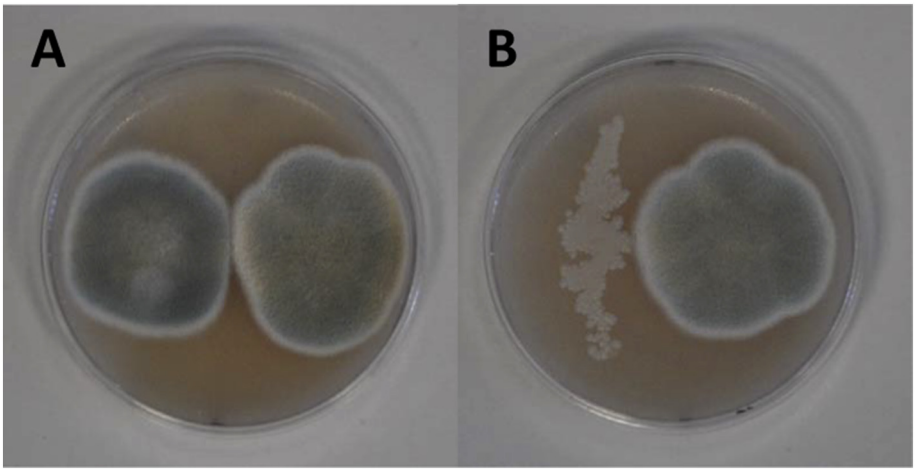

Fig. 2. Interaction assay between *P. griseofulvum* and protective cultures at day 15 of incubation on a dry-fermented sausage-based medium at 12 °C. A: *P. chrysogenum* (left) *vs P. griseofulvum* (right); B: *D. hansenii* (left) *vs P. griseofulvum* (right).

3.2. Interaction between P. griseofulvum and protective cultures

The strains inoculated on dry-fermented-sausage agar-based medium contacted the other ones at days 13–15 of incubation at 0.956 a_w. No interaction was observed at 0.924 a_w for the 18 incubation-days given that the growth was quite slow. Additionally, *P. acidilactici* was not able to grow at any a_w tested, then, no interaction data from this strain was obtained. The interactions between *P. griseofulvum* and *P. chrysogenum* or *D. hansenii* showed mutual inhibition on contact or space between colonies (interaction type B, < 2 mm) (Fig. 2). This has been rated as Numerical value 2. Then, both interactions between *P. griseofulvum* and the other two biocontrol agents can be rated as 2/2 as previously described (Magan and Lacey, 1984).

3.3. Protective cultures effect on fermented-sausage agar-based medium

The effect of *D. hansenii* and *P. chrysogenum* on the ability of *P. griseofulvum* to produce CPA was tested on dry-fermented-sausage agar-based medium. *P. griseofulvum* produced *c.a.* 12,000 ng/g CPA in this culture medium (Fig. 3). Every protective agent displayed a different effect on CPA produced by *P. griseofulvum*. *D. hansenii* did not affect CPA amounts whilst *P. chrysogenum* was able to reduce ($p \leq 0.01$) the CPA production under LOD. In addition, co-inoculation of both protective cultures was able to minimized CPA quantities produced by *P. griseofulvum*.

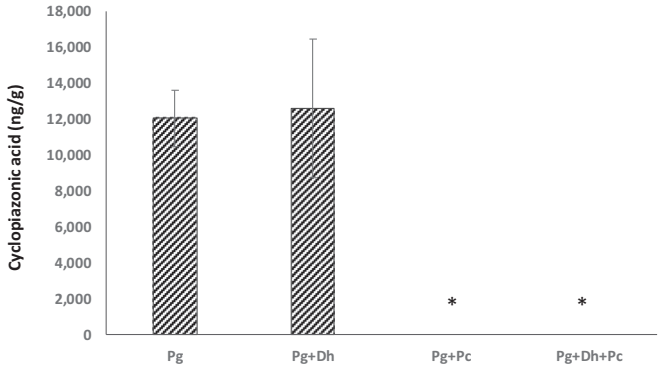

Fig. 3. Cyclopiazonic acid (CPA) production of *P. griseofulvum* (Pg) co-inoculated with biocontrol agents: *P. chrysogenum* (batch Pg + Pc), *D. hansenii* (batch Pg + Dh), both microorganisms together (batch Pg + Pc + Dh) and a control (batch Pg) on dry-fermented sausage-based medium. Plates incubated at 12 °C for 21 days. Bars indicate standard deviation of the means. Significant differences with the control (batch Pg) are indicated by an asterisk ($p \leq 0.05$).

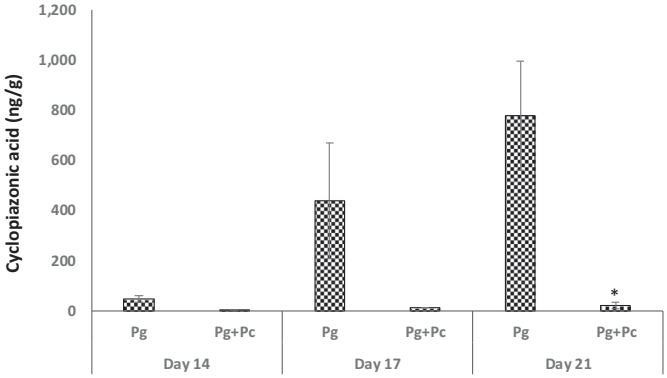

Fig. 4. Cyclopiazonic acid (CPA) production of the *P. griseofulvum* (Pg) and co-inoculated with *P. chrysogenum* (batch Pg + Pc) on dry-fermented sausages, ripened for 21 days. Sampling was taken at 14, 17 and 21 days. Bars indicate standard deviation of the means. Significant differences with batch Pg, for every sampling day, are indicated by an asterisk ($p \leq 0.05$).

3.4. Effect of protective cultures eon CPA production in dry-fermented sausage ripening

Fig. 4 shows the effect of *P. chrysogenum* on CPA production at days 14, 17 and 21 of dry-fermented sausage ripening. The CPA amounts were increasing from day 14 to day 21 in the batches Pg ($p \leq 0.05$). At day 21 of ripening, the dry-fermented sausages contained *c.a.* 800 ng/g CPA (Fig. 4). In the batches Pg + Pc, the CPA amounts encountered were lower than 20 ng/g and they were not increasing throughout the incubation time. In spite of the fact of the differences between batches, only at day 21 of incubation statistically differences were found ($p \leq 0.05$), being the reduction of CPA production provoked by the presence of *P. chrysogenum* around 40 times.

3.5. PgAFP gene expression

To study the mechanisms involved in the toxin inhibition by the protective agent *P. chrysogenum*, the expression of the *PgAFP* gene in *P. chrysogenum* was assessed. Both at 14 and 17 days, the batches co-inoculated with *P. chrysogenum*, showed *PgAFP*-gene expression. The absolute expression values of the PgAFP gene were 13.97 ± 0.95 and 18.2 ± 0.62 log copy numbers by days 14 and 17 of incubation.

4. Discussion

To the best of our knowledge, this is the first work which develops a successful biocontrol strategy against a CPA-producing *P. griseofulvum*. This work includes *in vitro* screenings performed with three biocontrol agents for obtaining an efficient protective culture to be used in dry-fermented sausages. In addition, several assays were conducted to look into the mechanisms involved in the inhibition of CPA production provoked by bioprotective agents.

Despite of OTA is the most concern mycotoxin in dry-cured meat products, there are other toxins which deserve to be studied. There are very few studies on the presence of CPA in meat products, due on the one hand, the CPA is an unstable molecule that can be degraded through chemical and physical processes (Diaz et al., 2010) and, on the other hand, little toxicity data on CPA relevant for risk assessment are available so far (Ostry et al., 2018). Despite all this, CPA has been found in salami (Ostry et al., 2018) and even in dry-cured ham (Alapont et al., 2014; Bailly et al., 2005; Peromingo et al., 2018b). Moreover, CPA has, by far, higher stability on dry-cured meat products in comparison with other common mycotoxins: patulin, OTA and citrinin (Bailly et al., 2005).

The mycotoxin production is linked to the substrate parameters, mainly a_w and temperature (Schmidt-Heydt et al., 2008). The *P. griseofulvum* strain used in this work has been found to produce the maximum CPA amount in dry-cured ham based-medium at 25 °C and 0.95 a_w (Peromingo et al., 2018a). The a_w of the dry-fermented sausages cannot be handly modified; however, the temperature is usually modified depending of the meat product and the desired ripening length (Bernáldez et al., 2013). The ripening temperature was set at the lowest one possible among those allowed for dry-fermented sausage ripening, in order to minimize the mycotoxin production. However, even at 12 °C, a great amount of CPA was detected in the final product. Interestingly, this amount dramatically increased in the last 7 days of incubation, even at the set relatively low temperature. This increment of CPA can also be due to the substrate, given that dry-fermented sausages undergo an intense proteolysis (Ordóñez et al., 1999), which releases free amino acids, such as tryptophan, a direct precursor of CPA (Chang et al., 2009; Liu and Walsh, 2009).

Therefore, it is required a strategy to counteract this potential hazard. The biological control is one of the best alternatives, although the biological agents should fulfil some requirements such as being non-toxigenic and isolated from dry-cured meat products. The three microorganisms tested as protective cultures against *P. griseofulvum* do not have potential toxigenicity and they were isolated from dry-cured meat products (Acosta et al., 2009; Núñez et al., 2015), even *P. acidilactici* supplied by Laboratorios Amerex. However, neither *P. acidilactici* nor *D. hansenii* successfully inhibited the CPA production. The bacterium did not grow in the dry-fermented sausages-based media assays. It is likely that the combination of low a_w values and temperatures avoided the *P. acidilactici* growth, then this bacterium was not selected for the next experiments conducted in this work. *D. hansenii* was able to grow in the dry-fermented sausage based-media, it displayed a mutual inhibition on contact or space between colonies small (< 2 mm) with *P. griseofulvum*. This yeast strain showed ability to produce volatile compounds and compete for space and nutrients, these facts led to *Penicillium verrucosum* growth inhibition as well as toxin production repression (Núñez et al., 2015). However, these assays performed in NaCl-rich agar plates for radial inhibition and in dry-fermented sausages slices incubated at higher temperatures (20 °C) which could explain the differences in growth and toxin inhibition.

The only bioprotective agent which achieved a successful CPA reduction was *P. chrysogenum*, both in plates and in the dry-fermented sausage ripening process. Among the tools to inhibit unwanted molds *P. chrysogenum* possesses the ability to produce the antifungal protein PgAFP, able to inhibit a wide range of molds (Delgado et al., 2015). However, not every mold is sensitive to this protein. In fact, *P. griseofulvum* could be considered as low sensitive, according to the inhibition rates observed, similarly to *P. griseofulvum* CECT 2919 (Delgado et al., 2015). The inoculation of the PgAFP producer allows a continuous PgAFP releasing, in contrast to the addition of a given quantity of PgAFP. The constant PgAFP synthesis has been proved by gene expression assessing, in a real ripening process, in comparison with those set for the *in vitro* production (Acosta et al., 2009). The interaction assay demonstrated that at those particular conditions, *P. chrysogenum* does not produce enough PgAFP amounts to inhibit the *P. griseofulvum* growth. However, on dry-fermented sausages *P. chrysogenum* produced PgAFP on the basis of the gene expression observed. It is not possible to measure the impact of the antifungal protein on the growth or CPA production by *P. griseofulvum*. Although it does not seem to be able to inhibit the growth, attending to the interaction assay, the amounts of PgAFP released may interfere in the CPA production. Also, the interaction with *P. griseofulvum* could provoke an overexpression of the *PgAFP* gene and the consequent PgAFP production; however, further studies are required to completely elucidate these mechanisms and achieve even a higher efficiency.

This work has demonstrated that the production of CPA in dry-fermented sausages cannot be only prevented by adjusting the technological parameters of the sausage using a relatively low temperature. The PgAFP protein-producing *P. chrysogenum* dramatically reduces CPA production on dry-fermented sausages. Given that the metabolites produced by *P. chrysogenum* are considered as GRAS (generally recognised as safe), the use of this strain should be considered together with good manufacturing practices in HACCP procedures for reducing the consumer's exposure to CPA due to dry-fermented sausages consumption.

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