Food Control 153 (2023) 109972

Contents lists available at ScienceDirect

Food Control

journal homepage: www.elsevier.com/locate/foodcont

Plant extracts as biopreservatives against *Penicillium nordicum* in dry-cured sausages

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ARTICLE INFO

Keywords:
Dry-cured sausages
Penicillium nordicum
Ochratoxin A
Plant extracts

ABSTRACT

One of the major hazards in cured meat is the ochratoxin A (OTA) accumulation, being the main producer *Penicillum nordicum*. Plant extracts can be used as biopreservative agents to its minimisation. The aim of this work was to evaluate the activity of essential oils from rosemary (REO), thyme (TEO) and oregano (OEO), and an acorn extract (AE) against three *P. nordicum* strains by analysing their inhibitory capacity on their growth and OTA biosynthesis in a dry-cured sausage-based agar. When evaluating their effect on the mould growth, the essential oils showed different antifungal activity, being concentration dependent. Concretely, the TEO and OEO activity was greater than that of REO, with a loss of activity throughout the incubation. As incubation time progressed, the plant extract ability to reduce the OTA content decreased. Additionally, differences at strain level were noticed depending on the plant extract, since some of them were able to trigger an overproduction of OTA. REO 500 μL/mL and OEO 50 μL/mL reduced the OTA production up to 93 and 98%, respectively, showing the best outcomes to be further explored as effective strategy to control the presence of OTA due to *P. nordicum* in dry-cured sausages.

1. Introduction

Dry-cured sausages represent one of the most important type of products commercialised by the European meat industry (Martín-Sánchez et al., 2011). During their ripening, moulds potentially producers of mycotoxins grow on their surface, being ochratoxin A (OTA) the most concerning one in dry-cured sausages (Berni et al., 2017; Iacumin et al., 2009; Markov et al., 2013). Thus, meat and its derived products are believed to be one of the most significant contributors to OTA exposure in the diet (Schrenk et al., 2020). Nevertheless, international guideline threshold levels for OTA in meat products has not been established yet, excepting that from the Italian Ministry of Health, which has set a limit of 1 μg/kg in meat and meat derivates (Ministerio della Sanità, 1999).

The most commonly found species with known ability to produce OTA in dry-cured sausages is *Penicillium nordicum* (Schmidt-Heydt et al., 2009). The application of different strategies against such ochratoxigenic mould to prevent OTA accumulation throughout maturation of dry-cured sausages has been deemed. Although the environmental conditions have been proposed as a mode of controlling the occurrence of OTA producing moulds in the meat industry (Rodríguez et al., 2014), it has to be taken into account that they normally favour the growth of moulds on the surface with the potential hazard of forming this mycotoxin. Consequently, other approaches are indispensable. Thus, legally permitted chemical preservatives, like natamycin, acid sorbic, sorbates and benzoates, can be used to prevent the unwanted mould growth in dry-cured sausages (European Commission., 2011; Silva & Cebola, 2016). However, these antifungal agents could cause allergies and side effects in some consumers (Martín-Sánchez et al., 2011), which has provoked the search for natural alternatives. There is also a growing attention of meat industry toward using natural ingredients to satisfy the strong consumers' demand for natural products. Therefore, essential oils have gained great popularity due to the fact that their antimicrobial activity, especially against a broad spectrum of bacteria, has been widely demonstrated (Burt, 2004). Their antiviral, antifungal, insecticidal and antioxidant properties have been documented too (Burt, 2004; Turek & Stintzing, 2013). Additionally, they are "generally recognised as safe"

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https://doi.org/10.1016/j.foodcont.2023.109972
Received 20 March 2023; Received in revised form 24 June 2023; Accepted 4 July 2023
Available online 5 July 2023
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(GRAS) for their intended use by the US Food and Drug Administration (FDA; Lysøe et al., 2006) as well as eco-friendly (Oliveira et al., 2018).

Among the essential oils' properties against toxigenic moulds, most of the studies have been focused on evaluating their inhibitory effects on ochratoxigenic Aspergilla. Accordingly, the potential role of different essential oils as biopreservative against *Aspergillus ochraceus, Aspergillus flavus* and *Aspergillus carbonarius* with application in several foodstuff has been reported (Basílico & Basílico, 1999; El Khoury et al., 2016; Hua et al., 2014; Lappa et al., 2017; Oliveira et al., 2018). Promising results have been similarly achieved when the antifungal activity of basil, oregano and caraway essential oils was evaluated in dry-cured sausages (Cenci et al., 2015; Kocić-Tanackov et al., 2020; Martín-Sánchez et al., 2011; Saggiorato et al., 2012), but they were not based on their anti-ochratoxigenic activity. Even though the main problem associated with the use of essential oils in such meat products is their effect on the sensory characteristics (Kocić-Tanackov et al., 2020), the superficial application of oregano essential oil (OEO) in dry-cured sausages did not negatively affect such properties and even seemed to shorten the time necessary for ripening, improving their texture (Martín-Sánchez et al., 2011).

The antifungal effect of other plant extracts different than essential oils has been poorly studied to date. Nonetheless, interesting results have been obtained when evaluating apple pomace and pomegranate peel extracts, and polyphenols extracted from olive mill wastewater against undesired moulds, including some toxigenic ones (Chaves-López et al., 2015; Li Destri Nicosia et al., 2016; Oleszek et al., 2019).

The aim of this study was to select from OEO, rosemary (REO) and thyme essential oils (TEO) and an acorn shell extract (AE) those with the most pronounced antiochratoxigenic action in a dry-cured sausage-based (FS) medium to get knowledge about their potential application as biopreservative strategy in meat derivates.

2. Material and methods

2.1. Chemicals

The pure acetone, acetic acid and magnesium sulphate were purchased from Scharlab S.L. (Spain). The acetonitrile HPLC Gradient Grade and the emulsifier Tween 80 were supplied by Fisher Bioreagents™ (Belgium). The used water was purified by passage through an EMD Millipore Milli-Q Integral Water System (Merck KGaA, Germany). Chemical standards for phenolic compounds were obtained from Sigma-Aldrich (USA) and Extrasynthese (Genay Cedex, France).

The commercial antifungal preparation 5BF.1654 (AP), including 8,3% of potassium sorbate and 2% of natamycin, was prepared in distilled water with 6% NaCl according to the manufacturer's instructions (Taberner S.A., Spain) and used as positive control in the growth assessment as well as in the evaluation of OTA biosynthesis.

OTA analytical standard to prepare working calibrant solutions was purchased from Sigma-Aldrich (Germany).

2.2. Preparation of plant extracts

Essential oils were extracted from oregano (*Origanum vulgare* L.), thyme (*Thymus vulgaris* L.) and rosemary (*Rosmarinus officinalis* L.) leaves. Extract was also prepared from the shell of acorn (*Quercus ilex* L.) fruits. Oregano and thyme were purchased from a local greengrocer in the city of Cáceres (Extremadura, Spain). Rosemary and acorns were manually collected in the region of Extremadura (Spain). The raw material was kept at −20 °C until extraction was carried out.

The essential oils were obtained by hydrodistillation using a Clevenger-type apparatus (Scharlab S.L.). A total of 200 g of the powdered material of each plant was placed in the round-bottom flask with 400 mL of distilled water and boiled for 2–2.5 h. The essential oils were then separated from the aqueous phase and stored at −20 °C in the absence of light until further use. Before using, the aqueous essential oil solutions were prepared employing Tween 80 (1% v/v).

Regarding the acorns, they were cleaned, sorted to eliminate damaged fruits and shelled. An amount of 40 g of ground shells was homogenised together with 80% (v/v) acetone using a BagMixer (Interscience™, France) for 3 min and then sonicated in an ultrasonic bath Ultrasons (J.P. Selecta, Spain) for 15 min. The homogenate was filtered through filter paper and centrifuged for 3 min at 3500 g using an Eppendorf centrifuge 5810 R (Germany). The solvent was then removed in a rotary evaporator (Rotavapor® R-210 R-210, BÜCHI Labortechnik AG, Switzerland) at 35–40 °C. The AE was filtered through a 0.22 μm pore size nylon membrane (RephiLe Bioscience Ltd., USA) and stored at −80 °C until use.

2.3. Characterisation of plant extracts

The chemical characterisation of the essential oils was determined by gas chromatography-mass spectrometry (GC-MS) using a gas chromatograph 6890 GC (Agilent Technologies, USA) equipped with a HP-5 column (5% phenyl-95% dimethylpolysiloxane: 30 m × 0.25 mm x 0.25 μm) and coupled to a mass spectrometer detector, 5975C (Agilent Technologies). The carrier gas was helium. The injector port was in the splitless mode and at 250 °C and the injection volume was 1 μL. The temperature program was isothermal for 2 min at 40 °C and then raised at the rate of 5 °C/min to 300 °C, and held for 5 min. The GC/MS transfer line temperature was 280 °C. The mass spectrometer operated in the electron impact mode with an electron energy of 70 eV, a multiplier voltage of 1650 V and collecting data at a rate of 1 scan/s over a range of *m/z* 50–400. The identification of the compounds was accomplished by comparing the experimental mass spectra with those of Wiley and the NIST/EPA/NIH libraries. Finally, the chromatographic peaks areas were expressed in arbitrary area units.

The determination and quantification of phenolic compounds in the AE was performed using a HPLC equipment following the method described by Ibern-Gómez et al. (2002) with slight modifications. For quantitative analysis, phenolic compounds were separated using a Shimadzu Prominence HPLC apparatus (Shimadzu Corp., Japan) equipped with a quaternary solvent delivery system (LC-20AD), a DGU-20AS online degasser, an SIL-20A autosampler, a RF-10A XL fluorescence detector and an SPD-M20A Diode Array Detector (HPLC-FLD-DAD). The columns were a reversed-phase Agilent Poroshell 120 SB-C18 column (150 × 4.6 mm, particle size 2.7 μm) and a guard column (10 × 4.6 mm) with the identical material. Solutions of 0.2% (v/v) aqueous trifluoroacetic acid (A) and acetonitrile with 0.2% (v/v) trifluoroacetic acid (B) were used as eluents. A gradient program was used, varying eluent B concentration from 7% (min 0) to 7% (min 5); 5–25 min, 7–18% B; 25–46 min, 18–34% B; 46–60 min, 34–65% B. The flow rate was maintained at 0.5 mL/min and the temperature of the column was kept at 30 °C. Prior to analysis, the AE was filtered with a 0.45 μm PVDF filter (Agilent Technologies). Monomeric phenolic compounds were quantified in five subclasses: hydroxybenzoic acids (as gallic acid equivalents, 280 nm), catechins (expressed as catechin equivalents; fluorescence detection λ_{ex} = 280 nm, λ_{em} = 322 nm), hydroxycinnamic acids (as caffeic acid equivalents, 320 nm), flavonols (as quercetin equivalents, 365 nm), ellagitannins (as tannic acid equivalents, 280 nm) and ellagic acid derivatives (as ellagic acid equivalents, 365 nm).

The total amount of procyanidins was quantified by an analytical HPLC method described by Ollé et al. (2011) with some modifications. Oligomeric and polymeric procyanidins were depolymerised in the presence of a nucleophilic agent (floroglucinol) in an acidic medium. A volume of 0.5 mL of the AE was evaporated to dryness under vacuum in a SpeedVac (Gyrozen, Korea) at 35 °C. The dried pellet was resuspended in 1 mL of ethyl acetate, transferred to a 4 mL glass flask and dried again under nitrogen.

The phloroglucinolysis reagent was prepared as follows. A 0.1M HCl methanol solution was firstly made (solution A). Solution B was then prepared by dissolving 500 mg of phloroglucinol in 10 mL of solution A.

Finally, solution C was made by dissolving 100 mg of ascorbic acid in 10 mL of solution B. The reaction started adding 1 mL of solution C to the AE, it was then stopped by placing the samples in an ice bath and diluting the reaction medium with 1.5 mL of a 40 mM ammonium acetate solution. The final solution was filtered through a PVDF 0.45 μm filter before HPLC assessment. The same chromatographic system and column employed for the identification of monomeric phenolic compounds were used for the detection of phloroglucinolysis products. Solutions of 0.1% (v/v) aqueous formic acid (A) and acetonitrile with 0.1% formic acid (B) were utilised as eluents. A gradient program was used, varying eluent B concentration from 7% (min 0) to 7% (min 5); 5–24 min, 7–18% B; 24–45 min, 18–34% B; 45–55 min, 34–65%. The flow rate was maintained at 0.5 mL/min and the temperature of the column was kept constant at 30 °C. The injection volumes were 3 and 1 μL for the analysis of procyanidins and the remaining compounds, respectively. Phloroglucinolysis products were characterised by their UV–vis spectra and retention time relative to external standards. A fluorescence detector (λ_{ex} = 280 nm, λ_{em} = 322 nm) was used simultaneously to improve the identification procedure. Total procyanidins was determined as the sum of the quantified subunits.

2.4. Mould strains

P. nordicum FHS15, CBS 323.92 and BFE856 from the Culture Collection of Food Hygiene and Safety at the University of Extremadura (Spain), Centraalbureau voor Schimmelcultures (The Netherlands) and Federal Research Centre for Nutrition and Food (Germany), respectively, were used in this research. All of them had been previously isolated from cured meat derivates and proven as ochratoxigenic.

2.5. Culture media and inoculum preparation

Yeast Extract Sucrose (YES) agar contained 20 g/L yeast extract (Scharlab, S.L.), 20 g/L bacteriological agar (Scharlab, S.L.) and 125 g/L of sucrose (Scharlab, S.L.). The FS agar was prepared using 25% (w/v) lyophilised and minced dry-cured fermented sausages "salchichón", according to Álvarez et al. (2020).

To obtain the inocula, the mould strains were 3-point inoculated on YES agar and incubated at 25 °C for 10 days. The spores were collected using 5 mL of sterile phosphate buffered saline (PBS; 0.32 g/L sodium dihydrogen phosphate anhydrous (Scharlab S.L.), 1.09 g/L di-sodium hydrogen phosphate anhydrous (Scharlab S.L.), 9 g/L NaCl (Fisher Chemical, USA)) and rubbing the surface with a sterile glass rod. The spores were preserved in glycerol solutions at −80 °C. New mould cultures were used for each experiment, whose spore levels were established using a cell counting chamber BLAUBRAND® Thoma pattern (Brand, Germany).

2.6. Effect of essential oils on the growth of ochratoxigenic moulds

The antifungal effect of the essential oils against the *P. nordicum* strains was determined by the standard agar disc diffusion technique. A volume of 100 μL of 10^7 spores/mL of each mould strain was individually spread on Petri plates containing YES agar. After the inocula were dried, sterile filter paper discs (4.5 mm diameter) were placed on the inoculated plates and impregnated with 10 μL of the plant extracts. Concentrations of 1000, 750, 500, 250, 100 and 50 μL/mL were tested for the three essential oils. Additionally, concentrations of 25, 20, 15, 10, 5 and 1 μL/mL were checked for TEO and OEO, due to the complete inhibition of the growth of the ochratoxigenic moulds by the initially evaluated ones. Two discs containing 50 μL (AP1) and 25 μL (AP2) of the AP were used as reference control. One disc impregnated with Tween 80 was prepared as untreated control. The plates were incubated at 12 °C for 8 days. The efficacy of treatments was evaluated at days 4 and 8 by measuring the diameter of the inhibition zones, which were expressed in millimeters (without including the diameter of the paper disc). Studies were performed in triplicate.

2.7. Effect of plant extracts on the OTA production

OTA biosynthesis by the three *P. nordicum* strains was evaluated in the FS agar. To simulate the manufacture of dry-cured sausages, a collagen casing (Viscofan, Spain) was placed on the surface of the FS agar contained in 5-cm diameter Petri plates. Formerly, the casing was immersed in ethanol (100% v/v) during 2 s and exposed to ultraviolet light in a laminar air flow hood (Telstar, Japan) for 24 h. The casing was then rehydrated and laid on the medium.

A volume of 50 μL from each selected extract concentration and 50 μL (10^7 spores/mL) from each mould strain were distributed in the casing surface. The essential oil levels were selected because of showing the highest efficiency in the previous assay (section 2.6). Thus, the concentrations of the essential oils finally used to determine their activity against OTA presence were as follows: 500 μL/mL and 250 μL/mL for REO (REO 500 and REO 250, respectively), and 50 μL/mL and 25 μL/mL for TEO and OEO (TEO 50 and OEO 50, and TEO 25 and OEO 25, respectively). Regarding AE, the most efficient concentrations were chosen from a previous study (data not published), being 20 μL (AE1) and 10 μL (AE2). As positive controls, the same spore solution concentration was inoculated together with 150 μL (AP3) or 50 μL (AP1) of the AP. Plates only inoculated with the studied moulds were used as untreated control.

The plates were incubated at 12 °C for 15 days to replicate a usual ripening process of dry-cured sausages. Samples were taken at 7 and 15 days for OTA synthesis analysis. The experiment was carried out in triplicate.

The OTA extraction from the mould mycelium including the casing was performed using the QuEChERS methodology described by Delgado et al. (2018). The analytical technique of liquid chromatography coupled to mass spectrometer (LC-MS/MS) was used for the mycotoxin quantification using the procedure previously reported by Álvarez et al. (2020). Its limit of quantification (LOQ) and limit of detection (LOD) were 0.5 ng/g and 0.20 ng/g, respectively.

The obtained results were expressed as percentage of OTA compared to the untreated control (mould grown in the absence of antifungal treatment, value = 100%).

2.8. Statistical analysis

Statistical analysis of the data was performed using the software SPSS v. 20.0. (IBM, USA). To statistically evaluate the differences in the mould growth between the extracts and the positive and untreated controls, the Student's *t*-test was applied, after assuming a normal distribution by using the Shapiro-Wilk test ($n < 30$). For the evaluation of statistical differences between different concentrations of the same plant extract, the ANOVA test was applied, followed by the Tukey post-hoc range test to identify homogeneous subsets of means of the different concentrations that do not differ from each other. Data on OTA production was tested for normality using the Shapiro-Wilk test. Since they failed such test, the non-parametric Kruskal-Wallis test was performed. The Mann-Whitney *U* test was then used to compare the mean values. The statistical significance was set at $p \leq 0.05$.

3. Results

3.1. Chemical characterisation of plant extracts

The essential oils' yield from the dried leaves was about 0.5%. A total of 43 compounds were identified when evaluating the three essential oils (Table 1). The greatest number of compounds (35) was detected in the TEO, with thymol (33.7%) and γ-terpinene (15.29%) as the most abundant components. A total of 25 compounds were found in REO, being camphor (29.71%) and eucalyptol (13.19%) the most prevalent

Table 1
Constituents of essential oils tested in this study determined by Gas Chromatography-Mass Spectroscopy (CG-MS) [a].

Compound	Identification [b]	Essential oils		
		Rosemary	Thyme	Oregano
2-Methylpentane	MS	4.27	6.35	4.43
3-Methylpentane	MS	48.68	69.04	49.80
Methylcyclopentane	MS	21.85	31.52	22.00
Methyl 2-methylbutyrate	MS	n.d. [c]	3.33	n.d.
α-Thujene	MS	n.d.	16.47	7.85
α-Pinene	MS	60.20	7.51	3.23
Camphene	MS	26.06	4.99	n.d.
Vinyl amyl carbinol	MS	n.d.	21.80	n.d.
β-Myrcene	MS	11.74	25.79	13.08
3-Octanone	MS	13.59	n.d.	n.d.
3-Octanol	MS	n.d.	2.81	n.d.
β-Pinene	MS	21.98	25.80	n.d.
α-Phellandrene	MS	n.d.	2.98	n.d.
β-Phellandrene	MS	n.d.	n.d.	2.90
***o*-Cymene**	MS	8.84	16.75	47.92
d-Limonene	MS	22.68	6.93	2.75
Eucalyptol	MS	94.60	17.97	5.66
Cis-beta-Ocymene	MS	n.d.	n.d.	3.32
γ-Terpinene	MS	4.07	186.21	88.58
Linalool	MS	18.16	60.84	n.d.
(+)-4-Carene	MS	4.01	31.71	18.56
Sabinene hydrate	MS	n.d.	43.70	n.d.
Camphor	MS	212.78	9.55	n.d.
Borneol	MS	38.94	25.45	n.d.
Terpinen-4-ol	MS	9.55	19.25	3.40
α-Terpineol	MS	17.95	6.17	3.76
Thymol methyl ether	MS	n.d.	19.12	n.d.
isothymol methyl ether	MS	n.d.	15.25	n.d.
Nerol	MS	n.d.	3.36	n.d.
Verbenone	MS	16.08	n.d.	n.d.
Thymol	MS	n.d.	409.85	201.64
p-Thymol	MS	n.d.	4.71	n.d.
Carvacrol	MS	n.d.	79.49	33.67
Borneol acetate	MS	21.62	3.78	n.d.
Methyleugenol	MS	2.66	n.d.	n.d.
***p-tert*-Butylcatechol**	MS	n.d.	2.69	n.d.
Caryophyllene	MS	13.20	22.87	4.39
Humulene epoxide	MS	2.81	n.d.	n.d.
Beta-Bisabolene	MS	n.d.	n.d.	4.58
1,4,7,-Cycloundecatriene, 1,5,9,9-tetramethyl-, Z,Z, Z-	MS	8.84	n.d.	n.d.
(.+/−.)-Cadinene	MS	n.d.	3.81	n.d.
Caryophyllene oxide	MS	10.91	5.26	n.d.
.tau.-Cadinol	MS	n.d.	4.74	n.d.

[a] Results are expressed in Arbitrary Area Units ($x10^{-6}$).
[b] Identification by Wiley and NIST/EPA/NIH mass spectrum libraries.
[c] n. d.: not detected.

ones. The OEO showed the lowest number of compounds (19) with thymol (38.66%) and γ-terpinene (16.98%) as the most abundant ones.

Once the presence of both monomeric phenolic compounds and the total content of procyanidins were evaluated and quantified, the latter were found to be the major compound in the AE (Table 2).

Table 2
Constituents of acorn extract (AE) tested in this study determined by High Performance Liquid Chromatography Fluorescence and Diode Array Detection (HPLC-FLD-DAD).

Phenolic profile	mg/100 mL of AE
Hydroxybenzoic acids	3.17 ± 0.10 [a]
Ellagitannins	6.19 ± 0.36
Hydroxycinnamic acids	0.49 ± 0.05
Catechins	66.27 ± 6.55
Flavonols	0.43 ± 0.02
Ellagic acid derivatives	3.61 ± 0.11
Procyanidins	234.85 ± 17.99
Total	315.02 ± 25.19

[a] Data are shown as mean ± SD.

3.2. Effect of essential oils on the growth

The inhibitory effects of the essential oils against *P. nordicum* are summarised in Figs. 1–3. Differences at mould strain, incubation time, and essential oil nature and concentration were obtained. Regarding the latter, the checked amounts of extracts presented inhibitory activity on the mould growth in a dose-dependent manner.

The initial range of concentrations evaluated in this work was the same for the three essential oils under study (1000-50 μL/mL). Since all those concentrations of OEO and TEO completely inhibited the growth of the tested moulds (data not shown), lower concentrations of OEO and TEO (25-1 μL/mL) were then checked (Figs. 1 and 2).

As expected, mould growth was not inhibited in the untreated control (Tween 80). The highest reduction in the growth of all ochratoxigenic strains was obtained for AP1 and AP2 at both incubation time. Additionally, significant differences between the antifungal capacity of such positive controls (AP1 and AP2) and the three essential oils were found.

Regarding OEO, the four highest concentrations (25, 20, 15 and 10 μL/mL) showed antifungal action against most of the studied strains at 4 days of incubation (Fig. 1A). However, no antifungal activity was revealed for any of the OEO levels at the end of the incubation (Fig. 1B). All the tested TEO concentrations, except the lowest ones (5 and 1 μL/mL), hampered the mould growth at 4 days of incubation (Fig. 2A). At the end of the incubation period, only the two highest TEO amounts (25 and 20 μL/mL) remained having significant antifungal activity against *P. nordicum* CBS 323.92 (Fig. 2B). Concerning REO, all its quantities inhibited the growth of the three tested moulds at 4 days of incubation (Fig. 3A), while only the highest REO concentration (1000 μL/mL) showed inhibition of all strains at the end of the incubation period (Fig. 3B).

Comparing the results obtained in the presence of TEO and OEO, great similarity is observed since the same essential oil concentrations were used. The main difference between them was that the two highest ones of TEO (25 and 20 μL/mL) still caused halo inhibition against strain *P. nordicum* CBS 323.92 (Fig. 2B), while it does not occur with OEO (Fig. 1B). In terms of the sensitivity of the mould strains tested, *P. nordicum* FHS15 was the most susceptible one at most of the tested three essential oil levels.

3.3. Effect of plant extracts on the OTA production

After evaluating the results from the previous screening (section 3.2), the antiochratoxigenic effect of the highest essential oil concentration provoking complete inhibition of the mould growth and the lowest one allowing the mould growth was studied (Fig. 4).

The OTA concentrations obtained by each of the 3 strains at 7 days ranged from <LOD to 214.04 ng/g, while the concentrations obtained at 14 days ranged from <LOD to 78.01 ng/g.

Although the findings revealed different patterns depending on the ochratoxigenic strain, *P. nordicum* FHS15 and BFE856 showed the most analogous ones (Fig. 4). In general, the inhibitory activity of the potential biopreservatives was higher at day 7 than at the end of the incubation period. Concretely, all the treatments significantly diminished the OTA generation in *P. nordicum* FHS15 and BFE856 at day 7 of incubation. Similarly, a reduction of OTA levels was detected for such strains in the presence of most of the treatments at day 15. Nevertheless, the addition of OEO 25 and AP3 significantly increased the OTA biosynthesis respect to the untreated control in *P. nordicum* FHS15 at the end of the incubation period. At day 15, the best outcomes were achieved with REO 500 and AE2 for *P. nordicum* FHS15 and with OEO 50 and AE2 for *P. nordicum* BFE856. Regarding *P. nordicum* CBS 323.92, its OTA production was not influenced by most of the potential biopreservatives at day 7. At day 15, TEO 50, TEO 25, REO 250, AE1, AE2

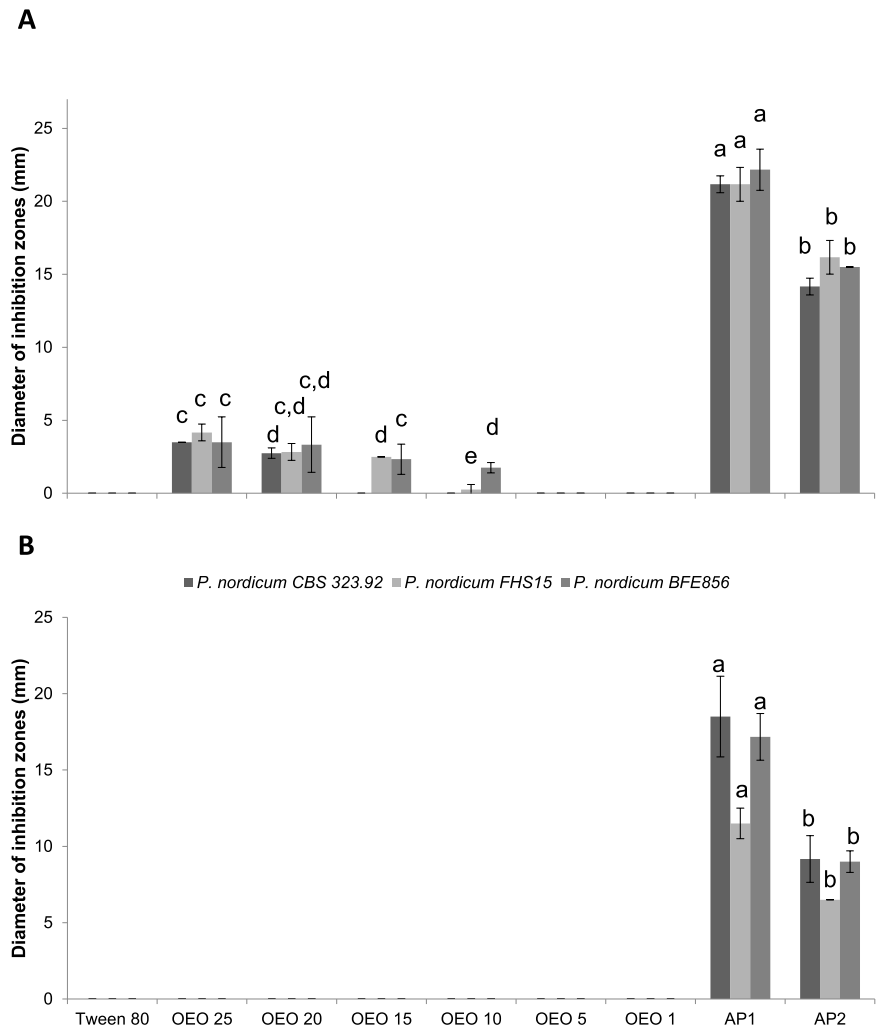

Fig. 1. Effect of different concentrations of oregano essential oil (OEO) on the growth inhibition of *Penicillium nordicum* FHS15, CBS 323.92 and BFE856 after incubation on YES agar for 4 (A) and 8 days (B). Treatments: 10 μL from each of the following ones: Tween 80 (1% Tween 80 v/v), OEO 25 (OEO at 25 μL/mL), OEO 20 (OEO at 20 μL/mL), OEO 15 (OEO at 15 μL/mL), OEO 10 (OEO at 10 μL/mL), OEO 5 (OEO at 5 μL/mL), OEO 1 (OEO at 1 μL/mL), AP1 (50 μL of the commercial antifungal compound), AP2 (25 μL of the commercial antifungal compound). The diameter of the inhibition zones (mm) is given as mean ± standard deviation of triplicate experiments. Statistical differences between all treatments are indicated by different letters ($p \leq 0.05$).

and AP3 significantly enhanced the OTA biosynthesis by *P. nordicum* CBS 323.92, while OEO 25 significantly stimulated the OTA generation at both sampling times. Considering all the results, the most suitable antiochratoxigenic agents would be REO 500 and OEO 50.

4. Discussion

The demand for nutritious, healthy and safe meat-based products is growing in modern societies, encouraging the industry to develop new technologies (Kalogianni et al., 2020). In this context, novel antifungal approaches for the replacement of those chemicals currently used in the meat industry are being extensively studied. Nonetheless, their effectiveness against the ochratoxigenic moulds growing on the dry-cured sausages remains scarce.

It is well known that plant extracts are characterised by a complex combination of compounds that vary in their amounts depending on the plant genetics, harvesting time, light, climatic and geographical conditions and extraction methods (da Silva Bomfim et al., 2015, 2020). This chemical composition of essential oils determines their biological activity, being mainly attributed to the most abundant components (Farag et al., 1989). Thus, some of the major components from each essential oil tested in this work are likely the responsible for their distinct effect on the *P. nordicum* strains. Additionally, the critical role of minor components of essential oils in their antimicrobial activity has been stated (Burt, 2004; Nikolić et al., 2015; Ribeiro-Santos et al., 2015).

Complete inhibition of the *P. nordicum* growth was achieved by the initial range of OEO concentrations (1000–50 μL/mL) evaluated in this work. This result was in line with the findings of El Khoury et al. (2016), who observed a 100% reduction of the *A. carbonarius* development due to 100 μL/mL of OEO. Nonetheless, these authors reported the complete inhibition of the *A. carbonarius* growth when using concentrations as low as 1 and 5 μL/mL of OEO. Likewise, Martín-Sánchez et al. (2011) described high growth inhibition of *Cladosporium sphaerospermum* and *Mucor racemosus* in dry-cured sausages when testing 1 mL/L of OEO, ten times lower than the OEO 10, the lowest concentration at which this essential oil exerted inhibition at day 4 in this work. Similar to the growth reduction noted in this work when 25 to 10 μL/mL of OEO were used (Fig. 1), a reduction in the *Penicillium* counts throughout the processing of the *samarella*, a Cypriot dry-cured meat product, has been indicated for 1 and 5% OEO (Ulusoy et al., 2018). The dominant compounds thymol and γ-terpinene have been also reported as major components of OEO when its antifungal effect has been tested (Chaves-López et al., 2012; Do Nascimento et al., 2020; Teixeira et al., 2013).

Regarding the OTA production, the FS agar with the sterile casing was used to simulate the process of obtaining dry-cured sausages in order to identify certain behaviors under strictly controlled conditions, which may be hidden in natural systems, as mentioned above. Both its increase and reduction respect to the untreated control were observed depending on the OEO amount and the mould strain. The promising decrease is consistent with previous works using 0.50 and 0.25%, and 1 and 5 μL/mL of OEO against *P. verrucosum* (Ozcakmak et al., 2017) and *A. carbonarius* (El Khoury et al., 2016), respectively.

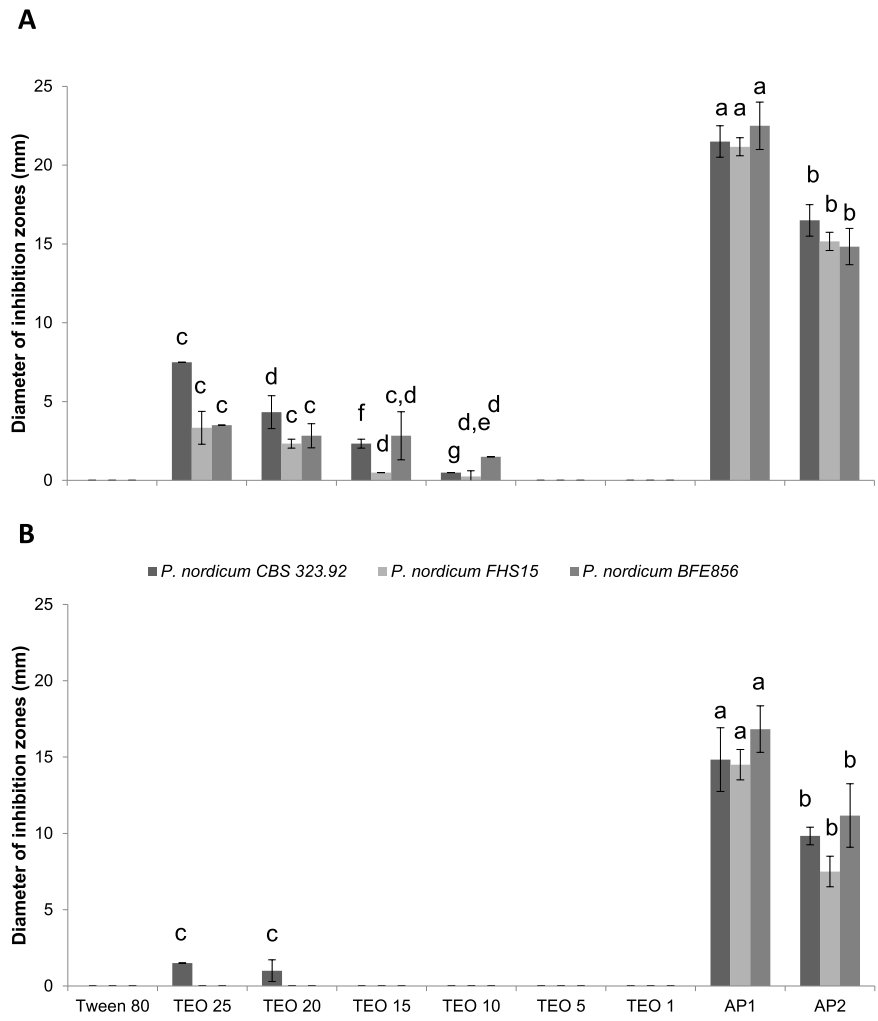

Fig. 2. Effect of different concentrations of thyme essential oil (TEO) on the growth inhibition of *Penicillium nordicum* FHS15, CBS 323.92 and BFE856 after incubation on YES agar for 4 (A) and 8 days (B). Treatments: Tween 80 (1% Tween 80 v/v), TEO 25 (TEO at 25 μL/mL), TEO 20 (TEO at 20 μL/mL), TEO 15 (TEO at 15 μL/mL), TEO 10 (TEO at 10 μL/mL), TEO 5 (TEO at 5 μL/mL), TEO 1 (TEO at 1 μL/mL), AP1 (50 μL of the commercial antifungal compound), AP2 (25 μL of the commercial antifungal compound). The diameter of the inhibition zones (mm) is given as mean ± standard deviation of triplicate experiments. Statistical differences between all treatments are indicated by different letters ($p \leq 0.05$).

The results also indicated the effectiveness of TEO for controlling the *P. nordicum* development, specially at the highest tested concentrations (1000–50 μL/mL). Daferera et al. (2000) reported analogous findings since a complete inhibition of the *P. digitatum* growth was observed at concentrations of 250–400 μL/mL of TEO. Correspondingly, Omidbeygi et al. (2007) demonstrated the intense action of TEO against *A. flavus* species, reaching the maximum activity at very low concentrations (0.50 and 0.35 μL/mL). The major components of TEO were thymol and y-terpinene, which is in line with previous studies about its antifungal effect (Oliveira et al., 2018; Omidbeygi et al., 2007). In addition, the biopreservative effect of thymol against 11 moulds has been examined being shown high inhibitory activity against *Cladosporium* spp. and *Aspergillus* spp. (Abbaszadeh et al., 2014).

Regarding the TEO impact on OTA production, several authors have reported its efficacy on reducing the mycotoxin accumulation as observed with TEO 25 and TEO 50 for two of the strains studied in the present work (Fig. 4A and C). Concretely, a decrease of 90% of the OTA amount produced by *A. carbonarius* was displayed when using 1 μL/mL of TEO after 4 days on incubation in a synthetic grape medium (El Khoury et al., 2017). Similarly, Socolic-Mihalak et al. (2012) obtained an OTA decrease by up to 60% when evaluating TEO concentrations between 0.625 and 2.500 μL/mL against *A. carbonarius*.

The antifungal action of REO observed in this study was consisting with previous reports evaluating its usefulness against *Penicillium* sp. (Cenci et al., 2015), since REO (1000 μL/mL) showed an inhibitory effect, although the lowest concentrations did not inhibited the development of the assayed moulds. The latter outcome differs from our work, where 50 and 500 μL/mL of REO still hampered their growth at 4 and 8 days, respectively. Such difference could be due to the different predominant compounds of REO in both studies. In fact, the major component of that REO used by Cenci et al. (2015) was eugenol, while camphor and eucalyptol were the main ones in the extract applied in the present work. Both of the latter compounds have shown various antimicrobial properties (Bouyahya et al., 2017; Ed-Dra et al., 2020; Rashed et al., 2021). Daferera et al. (2000) also established that REO, containing eucalyptol and camphor among its major active compounds, presented moderate inhibitory activity on the radial growth of *Penicillium digitatum*.

Despite REO 250 did not completely diminish the development of the three tested *P. nordicum* strains, its antiochratoxigenic effect was found for two of them (Fig. 4A and C). This finding is in line with a previous study where 1 and 5 μL/mL of REO showed a slight impact on the *A. carbonarius* growth but a significant impact on OTA production, being lowered 53.7% and 78.3%, respectively (El Khoury et al., 2016).

To the best of our knowledge few studies have been carried out to evaluate the effect of AE on ochratoxigenic moulds. Only studies based on its effect on the growth of different fungal species, such as *Tricophyton verrucosum* (Akroum, 2017), *Fulvia fulvum* (Karioti et al., 2011) and *Candida albicans* (Güllüce et al., 2004), have been performed. Regarding its composition, a great variability in terms of the major phenolic compounds among acorn species has been reported (Vinha et al., 2020). As obtained in this work, catechins turn out to be one of the most abundant compounds both in the acorn peel extracts analysed and from other parts of the oak tree (Cava et al., 2015). Similar to this study, the

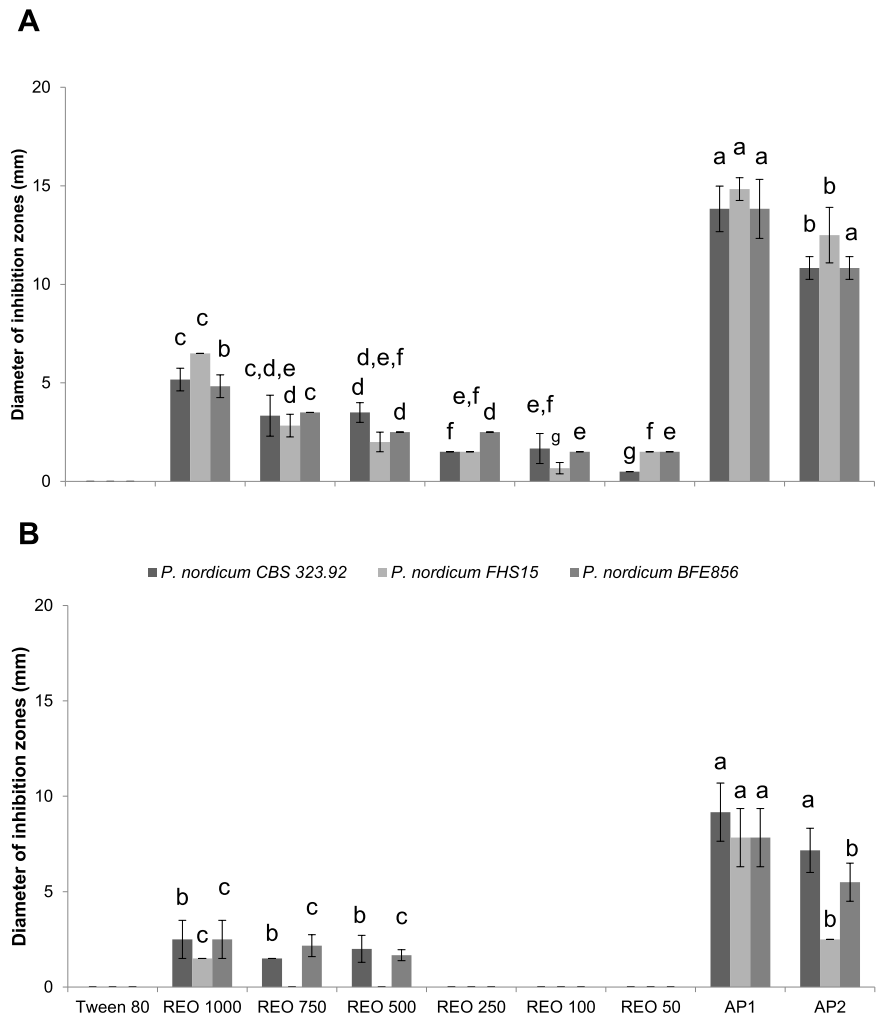

Fig. 3. Effect of different concentrations of rosemary essential oil (REO) on the growth inhibition of *Penicillium nordicum* FHS15, CBS 323.92 and BFE856 after incubation on YES agar for 4 (A) and 8 days (B). Treatments: Tween 80 (1% Tween 80 v/v), REO 1000 (REO at 1000 μL/mL), REO 750 (REO at 750 μL/mL), REO 500 (REO at 500 μL/mL), REO 250 (REO at 250 μL/mL), REO 100 (REO at 100 μL/mL), REO 50 (REO at 50 μL/mL), AP1 (50 μL of the commercial antifungal compound), AP2 (25 μL of the commercial antifungal compound). The diameter of the inhibition zones (mm) is given as mean ± standard deviation of triplicate experiments. Statistical differences between all treatments are indicated by different letters ($p \leq 0.05$).

presence of procyanidins, tannins and ellagitannins, gallic and ellagic acid, as well as their derivatives, is reflected in acorns when examining their antimicrobial activity (Huang, Niu, Li, Li, & Wang, 2008; Lee et al., 2020; Meyers et al., 2006; Papoti et al., 2018; Vinha et al., 2016).

Although AP was effective in reducing the mould development in the earliest stage, an increase of OTA presence was detected at the end of the incubation. On the contrary, Álvarez et al. (2020) found a significant decrease in OTA at 14 days of incubation respect to 7 days when evaluating the effect of 25 μL of AP, an amount slightly lower than AP1 and AP3 used in the present study, against *P. nordicum* CBS 323.92. Generally, AP was not more effective than essential oils, which is consistent with a previous work showing a greater antimicrobial activity from rosemary extract than that of certain common food additives, such as butylated hydroxytoluene (BHT; Ribeiro-Santos et al., 2015; Romano et al., 2009). As seen in previous studies, the use of certain commercial antifungal agents can lead to a decrease in the mould growth but triggers an increase in the mycotoxin production (Schmidt-Heydt et al., 2013). Therefore, it is necessary to ensure that an antifungal compound does not have side effects, such as increased production of mycotoxin, which are much more harmful than the mould growth (Schmidt-Heydt et al., 2013).

On the other hand, differences at the strain level were obtained, being *P. nordicum* FHS15 and BFE856 the most similar to each other in terms of OTA biosynthesis (Fig. 4). This coincides with the results obtained by Bernáldez et al. (2018) and Rodríguez et al. (2014), who studied the OTA generated by different strains of *P. nordicum* including CBS 323.92. Therefore, the direct proportional link between the incidence of ochratoxigenic moulds and the quantity of OTA is not clear (Álvarez et al., 2021a,b).

Finally, it is worth noting the relationship between the synthesis of OTA and the amount of the essential oil, being noted a decrease in the OTA produced by the three studied strains at the highest concentrations. This matches with the results achieved in other studies where a clear connection between the increasing concentration of essential oils and the decrease in the presence of OTA was obtained (Carmo et al., 2008; El Khoury et al., 2017; Hillen et al., 2010; Hua et al., 2014; Soylu et al., 2006). Furthermore, a loss of OEO activity in terms of OTA inhibition against the three strains was observed after 15 days of incubation. The loss of inhibitory capability of the three essential oils is consistent with other authors, who concluded that particular active compounds of essential oils have a reversible effect over time because of their oxidation (Hua et al., 2014; Hădărugă et al., 2014). This could be solved by re-supplementation with plant extracts or their co-inoculation with other biopreservative agents. The increase in OTA presence as incubation time progresses found in all three essential oils does not coincide with the study by Álvarez et al. (2020), based on the use of the same three plants but not their essential oils, who concluded with a reduction in OTA production under all treatments during the incubation time.

The enhancement of the OTA production observed for the *P. nordicum* strains in the presence of each of the treatments (Fig. 4) has been previously attributed to the stress imposed by the antifungal agent that could encourage the mycotoxin synthesis as a defense response (Alcano et al., 2016; Álvarez et al., 2020; D'Mello et al., 1998; Lappa et al., 2017; Schmidt-Heydt et al., 2009). In this line, it is worth

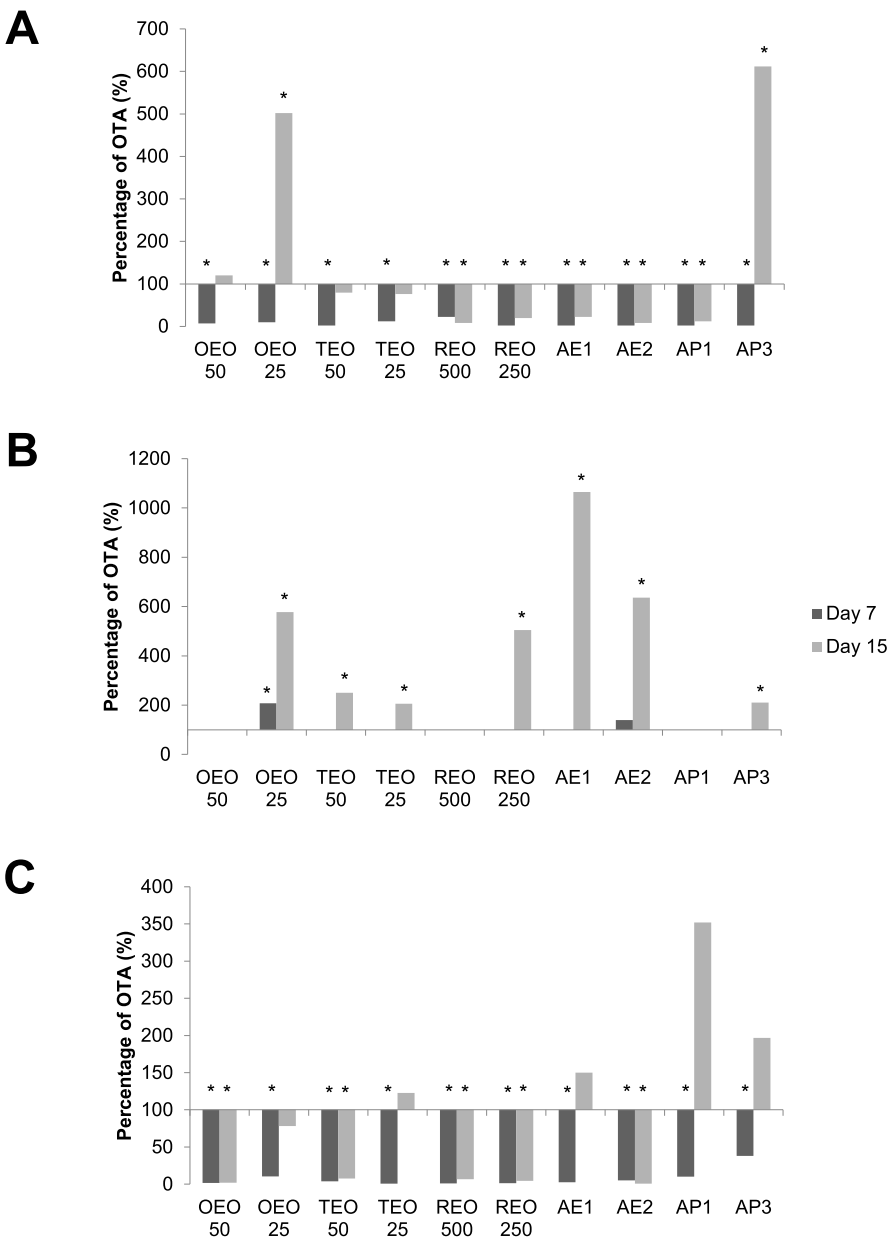

Fig. 4. Ochratoxin A (OTA) amount produced by *Penicillium nordicum* FHS15 (A), CBS 323.92 (B) and BFE856 (C) grown during 15 days at 12 °C in a dry-cured sausage-based agar with the addition of different plant extracts (oregano (OEO), rosemary (REO), thyme (TEO) and acorn (AE)) and a commercial antifungal preparation (AP). It is expressed as percentage of OTA compared to the untreated control (mould grown in the absence of antifungal compounds, value = 100%). Samples were taken at days 7 and 15 of incubation.
Treatments: 50 μL from each of the following ones: OEO 50 (OEO at 50 μL/mL), OEO 25 (OEO at 25 μL/mL), TEO 50 (TEO at 50 μL/mL), TEO 25 (TEO at 25 μL/mL), REO 500 (REO at 500 μL/mL), REO 250 (REO at 250 μ/mL), AE1 (20 μL of AE), AE2 (10 μL of AE), AP1 (50 μL of the AP), AP3 (150 μL of the AP). The statistical analysis was performed using the mean concentration ± standard deviation of each treatment. Statistical differences between the non-treated control (100%) and treatments are indicated by an asterisk ($p \leq 0.05$).

mentioning that the mould growth inhibition does not always entail a mycotoxin reduction biosynthesis, but sometimes just the opposite. This fact pushes for a comprehensive evaluation of multiple mould strains, growth and OTA assessment, as those performed in this work.

The obtained results reveal the importance of continuing research on the use of essential oils and other plant extracts like biopreservative agents in the meat industry as an alternative to commercial antifungals to consume increasingly less processed products. On the other hand, essential oils possess beneficial qualities for human beings, such as antioxidant, anti-inflammatory, antimicrobial and anticancer activity. In addition, TEO and REO have been used as medicines for centuries in a wide variety of places and are described in international and national pharmacopoeias (Sharifi-Rad et al., 2017).

Among other important consequences of the use of essential oils in the meat industry is the variation in the sensory characteristics of the products. Specifically, Martín-Sánchez et al. (2011) concluded that the use of OEO did not significantly affect negatively the sensory characteristics of sausages, such as odour, colour and juiciness, although, it impacted on hardness, despite positively. In addition, the use of essential oils rich in bioactive compounds, such as thymol, eucalyptol, γ-terpinene and camphor, in concentrations below 3% had no negative effects on the acceptance of some meat products (Djenane et al., 2011; García-Díez et al., 2016; Hayouni et al., 2008).

5. Conclusions

This was the first study based on using oregano, rosemary and thyme essential oils and acorn extract for controlling the production of OTA by three different *P. nordicum* strains in dry-cured sausages. The treatments based on rosemary and oregano essential oils at concentrations of 500 μL/mL and 50 μL/mL, respectively, were confirmed to be promising options as biopreservatives to prevent the OTA biosynthesis and replace the use of traditional antifungals in the meat industry. They are thus supposed to constitute an innovative tool integrated into meat systems to satisfy the ever-increasing demands for natural, quality, safe and healthy food. Future studies should be focused on the influence of

extract addition on safety and sensory properties of real matrix before any application at an industrial level.

Fundings

The present work has received funding by IB16045 project and GR18056 grant (*Junta de Extremadura-Consejería de Economía, Ciencia y Agenda Digital-, Fondo Europeo de Desarrollo Regional-"Una manera de hacer Europa"*).

CRediT authorship contribution statement

Elia Roncero: Formal analysis, Investigation, Methodology, Validation, Data curation, Visualization, Writing – original draft. **Josué Delgado:** Conceptualization, Supervision, Writing – review & editing. **David Morcuende:** Data curation, Investigation, Writing – review & editing. **Antonio Silva:** Data curation, Investigation. **María J. Andrade:** Conceptualization, Funding acquisition, Methodology, Project administration, Resources, Supervision, Writing – review & editing.

Declaration of competing interest

The authors declare that they have no known competing financia! interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

Acknowledgements

The authors wish to thank A. Rodríguez for her assistance when analysing the effect of essential oils in the growth of ochratoxigenic moulds. R. Geisen is acknowledged for the *P. nordicum* BFE856 donation.

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