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Osmotic stress as an adaptation mechanism of ochratoxigenic moulds in a dry-cured ham model system

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ABSTRACT

New strategies in the dry-cured ham industry advocate reducing NaCl or replacing it with KCl to improve consumer health without compromising safety. We examined the growth, ochratoxin A (OTA) production, and osmoadaptive responses of two common ham-colonising fungi, *Penicillium nordicum* and *Aspergillus westerdijkiae*, under four culture conditions: control (no added salt), 100 g/L NaCl, 100 g/L KCl, and 50 g/L each of NaCl + KCl. Media were based on lyophilized dry-cured ham, and strains were incubated at 25 °C for 12 days. High adaptation to salt-rich conditions was demonstrated by the faster development of both moulds in salt-supplemented media, with *A. westerdijkiae* exhibiting overall higher growth and OTA production. The unsupplemented control medium produced the greatest OTA levels, indicating that mild osmotic stress may maximise toxin synthesis. Intracellular glycerol assays revealed HOG-pathway activation in *A. westerdijkiae* under salt stress, whereas *P. nordicum* showed reduced glycerol accumulation, implying alternative adaptation routes. Free-chlorine release occurred via OTA and free chlorine in both species, though no direct correlation with OTA levels was found. Total NaCl replacement by KCl did not significantly alter OTA synthesis, suggesting feasibility for sodium reduction in dry-cured ham. However, partial NaCl substitution may risk elevated OTA production by *A. westerdijkiae*. These species-specific findings underscore the need for tailored mitigation strategies to ensure food safety in low-sodium dry-cured ham.

1. Introduction

Spanish dry-cured ham is a traditional meat product obtained after months or even years of ripening. During the first steps of the processing, the salting and drying are indispensable to reduce the risk of the presence of pathogenic bacteria and microbial spoilage (Martín et al., 2010). NaCl decreases the product water activity (a_w), contributing to microbial development suppression (Vidal et al., 2021). Additionally, NaCl exposes microorganisms to osmotic stress, resulting in a loss of water from the cell, causing microbial death or limiting their growth. Despite this, the environmental conditions of the manufacturing rooms favour mould growth on the surface of dry-cured hams due to their adaptation to xerophilic and halophilic conditions (Martín et al., 2004; Susca et al., 2017). The presence of filamentous fungi on this product improves the sensory quality of dry-cured ham by promoting proteolysis and the

typical flavour (Susca et al., 2017). However, mould development may also lead to the accumulation of toxigenic secondary metabolites as mycotoxins when the environmental and nutritional conditions are propitious (Pizzolato Montanha et al., 2018; Rodrigues et al., 2019; Rodríguez et al., 2012).

The most concerning mycotoxin in dry-cured meat products is ochratoxin A (OTA), which is commonly found in dry-cured hams from different European countries, including Spain, Portugal, and Croatia (Rodrigues et al., 2019; Rodríguez et al., 2012; Vulić et al., 2016). The last opinion released by the European Food Safety Authority (EFSA) about the risk of OTA in food concluded that preserved meat is one of the most important contributors to chronic dietary exposure to this mycotoxin in certain countries (Schrenk et al., 2020). OTA is mainly produced in dry-cured meat products by *Penicillium nordicum* (Coton et al., 2021; Iacumin et al., 2011, 2009), although another species, such as *Aspergillus*

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westerdijkiae has been shown to produce high OTA levels in dry-cured ham (Rodríguez et al., 2019), well over the limit established by Italian legislation, 1 µg/kg (Ministero della Sanità, 1999). The production of this mycotoxin could contribute to the adaptation of the mould to NaCl-rich habitats such as Iberian ham (Delgado et al., 2018; Schmidt-Heydt et al., 2008). Thus, it is of utmost interest to know the response of OTA-producing moulds toward the osmotic stress caused by the high NaCl content in dry-cured ham and its potential role in the rise of mycotoxin accumulation. In fact, it has been suggested that the constant biosynthesis of OTA in *P. nordicum* and *Penicillium verrucosum* ensures the excretion of chlorine out of the cell to maintain homeostasis in a rich NaCl substrate (Schmidt-Heydt et al., 2012). In this regard, the impact of NaCl concentration on *A. westerdijkiae* adaptation to the substrate or OTA production has not been studied so far, even though this species can produce high OTA levels on dry-cured ham (Rodríguez et al., 2019).

In addition, some authors have demonstrated that the High Osmolarity Glycerol response pathway (HOG) is activated in toxigenic moulds in response to osmotic stress (Rodríguez et al., 2016; Schmidt-Heydt et al., 2013). These studies have shown the different adaptations of *P. verrucosum* and *P. nordicum* to NaCl-rich habitats through the induction of the protein HOG1 and the expression of the gene (*Hog1*) that encodes it (Rodríguez et al., 2016; Schmidt-Heydt et al., 2013). This leads to an increase in intracellular glycerol and the restoration of turgor pressure to cope with the osmotic stress (Rodríguez-Peña et al., 2010). Furthermore, this pathway is also related to the fungal response to oxidative and temperature stresses, and it has also been associated with the cell wall integrity (CWI) pathway (Víglaš and Olejníková, 2020).

On the other hand, in order to prevent cardiovascular diseases, new trends in consumer profiles support lowering the amount of NaCl in food (Xiao et al., 2021). With the aim of reducing dietary Na⁺, the most usual strategy is to substitute other salts, primarily KCl, but also CaCl₂ and MgCl. While some authors have assessed the technological and sensory aspects of substituting other salts for NaCl in dry-cured ham (Armenteros et al., 2012; Ripollés et al., 2011), their effects on the adaptation response of ochratoxigenic moulds have been examined only in *P. verrucosum* in YES medium (Andrade et al., 2019), not in *P. nordicum* or *A. westerdijkiae* using a dry-cured meat-based matrix.

Thus, this study aimed to evaluate the growth and OTA production of *P. nordicum* and *A. westerdijkiae* on a dry-cured ham-based medium in the presence of NaCl and KCl, both separately and in combination, as well as their adaptive responses to the osmotic stress by producing free chlorine and glycerol.

2. Material and methods

2.1. Mould strains and inocula preparation

The ochratoxigenic moulds *P. nordicum* FHSCC Pn15 and *A. westerdijkiae* MUM. 16.142, both isolated from dry-cured ham, were used in this study. *P. nordicum* belongs to the Culture Collection of Food Hygiene and Safety of the University of Extremadura (Cáceres, Spain) and *A. westerdijkiae* to the Micoteca da Universidade do Minho (Braga, Portugal).

The ochratoxigenic moulds were inoculated in three points on potato dextrose agar (PDA) and incubated for 10 days at 25 °C. After this period, the spores were collected by scraping the colonies' surface and diluting them in phosphate-buffered saline [0.32 g of NaH₂PO₄ (Scharlab, S. L., Barcelona, Spain), 1.09 g of Na₂HPO₄ (Scharlab, S.L.), 9 g of NaCl, 1 L of distilled water]. The spores were quantified using a Thoma counting chamber Blaubrand® (Brand, Germany), and the inoculum concentration was adjusted to 10⁶ spores/mL.

2.2. Experimental settings

Using lyophilised dry-cured ham and varying concentrations of NaCl and KCl, four culture media were made. The medium control (C) was

elaborated with 30 g/L of lyophilised dry-cured ham and 20 g/L of bacteriological agar (Scharlab, S.L.). The medium NaCl was elaborated by adding 100 g/L of NaCl to medium C. By adding 100 g/L of KCl to medium C, the medium KCl was prepared. Finally, 50 g/L of each salt was added to medium C to create the medium NaCl+KCl. The culture media were autoclaved for 20 min at 121 °C. A LabMaster-a_w device (Novasina AG, Lachen, Switzerland) was used to measure the a_w values.

Before inoculation, sterile cellophane overlays (A. A. Packaging Limited, Preston, UK) were placed onto the media to avoid the contamination of the mycelium samples with the culture medium. Two µL from the mould inoculum (Section 2.1) were then plotted on the cellophane in the centre of the plate. For each treatment, the experiment was performed as three independent biological replicates (*n* = 3), with each replicate consisting of a separate plate. The plates were incubated for 12 days at 25 °C.

2.3. Growth assessment

The diameter of the mould colonies was daily measured in two perpendicular directions to determine the maximum growth rate (µ_m, in mm of radius/day). The radii of the colonies were plotted against each incubation time. After a lag phase, µ_m was obtained from the slope of the regression line following the methodology described by Álvarez et al., 2020.

2.4. Mycotoxin production

Following the method described by Delgado et al. (2018), 2 g of agar from each plate that coincided with the centre of fungal colonies from one of the colony diameters was collected after incubation and stored at -20 °C until mycotoxin extraction. The ochratoxin B (OTB) and OTA (both supplied by Sigma Aldrich, Madrid, Spain) were analysed by a Q-Exactive Plus (Thermo Fisher Scientific) coupled to a Dionex Ultimate 3000 RSLCnano (Thermo Fisher Scientific). The column was an Acclaim PepMap RSLC C18, 2 µm, 100 Å, 75 µm i.d. × 50 cm, nanoViper (Thermo Fisher Scientific). The eluent A employed was water:formic acid (0.1 %), and the eluent B acetonitrile:formic acid (0.1 %). The gradient used was 35–95 % Eluent B for 10 min, with a pre- and post-conditioning for 10 min with 10 % of Eluent B. The injection volume was 2 µL at a flow of 0.4 µL/min. The total run time was 15 min. Every analysis was carried out in full scan mode with a resolution of 70,000 full width at half maximum (FWHM). An ESI source (HESI II, Thermo Fischer Scientific) operating in positive ion mode (ESI⁺) was used. Each mycotoxin was identified by its retention time and its exact mass (*m/z*). OTB was eluted by 6.98 min and OTA by 7.74 min. Qual browser software belonging to Xcalibur v.3.1. (Thermo Fisher Scientific) was used to process the data. The limits of quantification (LOQ) were 0.062 and 0.125 ng/mL for OTA and OTB, respectively. The limits of detection (LOD) were 0.02 ng/mL for OTA and 0.042 ng/mL for OTB, respectively. They were calculated as the lowest evaluable concentration level at which the qualifier ion signal exceeds the noise level by factors of 3.5 and 10, respectively.

2.5. Intracellular glycerol determination

After incubation time, each colony was divided into two identical halves, one of which was taken out for glycerol determination and the other for free chlorine determination (see section 2.6). Prior to glycerol extraction, mycelia were dried at 60 °C for 24 h, weighed, and stored at -20 °C. The samples were then homogenised using a FasPrep-24 (MP Biomedicals LLC, Solon, OH, USA) with 5 steel beads of 3 mm in diameter at 4 m/s for two minutes after the mycelium had been resuspended with 300 µL of distilled water and a steel bead. The mycelia were then sonicated in a Branson sonifier™ 250 (Emerson, Spain) and centrifuged at 20800g for 15 min. Ten µL from the supernatant were transferred to another Eppendorf tube, and the intracellular glycerol was determined using the Glycerol Assay kit (Sigma-Aldrich, St. Louis,

Missouri, USA) following the manufacturer's instructions. To build a standard curve, different patterns of glycerol were used (1, 0.6, 0.4, 0.05, 0.03, and 0.015 mM). The standard curve showed excellent linearity ($R^2 > 0.99$). Blank was measured using distilled water instead of glycerol standards. One hundred μL of the reaction buffer was added to each sample and pattern, and the absorbance was measured at 570 nm by using the Varioskan LUX spectrophotometer (Thermo Fisher Scientific, Waltham, Massachusetts, USA).

2.6. Free chlorine determination

The mycelia portion used for free chlorine determination was washed twice with double-dionized water, recovered with a Miracloth filter (Calbiochem, Darmstadt, Germany), placed into a test tube, and also dried at 60 °C in a drying oven for 24 h, weighed, and then calcined in a muffle furnace at 700 °C. After that, the ashes were diluted in 1 mL of distilled water, and these contents were transferred to new Eppendorf tubes (Eppendorf, Hamburg, Germany). The tubes were stored at -20 °C until the Cl^- extraction by using the Chlorine Test Kit CD-1 (Aqualytic, Dortmund, Germany) following the manufacturer's instructions. Blank was measured using distilled water.

2.7. Statistical analyses

Statistical analysis was performed using the IBM SPSS Statistics 22.0 software (IBM, Armonk, New York, USA). The Shapiro-Wilks test was used to assess the normality of the data. As the data did not follow a normal distribution, the non-parametric Kruskal-Wallis test was used to determine if significant differences existed among groups for a_w , growth rate, mycotoxin concentration, and chlorine and glycerol determination. When a significant difference was found ($p \leq 0.05$), post-hoc pairwise comparisons were conducted using the Mann-Whitney U test to identify which specific groups differed. The Spearman's correlation test was also carried out between both mycotoxins, the growth, and the chlorine and glycerol contents. The statistical significance was established at $p \leq 0.05$.

3. Results and discussion

Excessive consumption of Na^+ is associated with a rise in cardiovascular illnesses that are linked to high blood pressure. In response to the increase in such diseases, the dry-cured ham industry is looking for new strategies to favour consumers' health. The physicochemical properties of dry-cured meat products may be affected when NaCl is substituted by KCl. From the perspective of food safety, the effects of these differences on the ability of toxigenic moulds to adapt and the formation of OTA during the processing of dry-cured ham are of utmost importance. This work compiles the first approach to understand how *P. nordicum* and *A. westerdijkiae* adapt to KCl, the most widely used NaCl alternative in the food industry.

3.1. Culture medium characteristics and growth assessment

When the a_w values of the three modified culture media (NaCl, KCl, and NaCl+KCl) were measured, no significant differences were found between them ($p = 0.36$), resulting in a unified mean a_w value of 0.94 in all cases. No differences in this parameter have previously been described in YES agar by the addition of NaCl or KCl (Andrade et al., 2019). These results indicate that the mould behaviour will depend on the kind of salt and not on the a_w value when comparing the salt-modified media. The different physiological responses can be due to the ionic stress exerted by Na^+ and K^+ . For example, Na^+ disrupts certain metabolic pathways, making the ion poisonous to cells and competing with K^+ for essential binding sites within the cells (Boumaaza et al., 2022). As expected, the control medium (C), which has no more solute added than those from the 3 % contained in the lyophilised dry-

cured ham, showed a significantly higher a_w of 0.97.

Fig. 1 displays the μ_m values for both toxigenic moulds. In comparison to *P. nordicum*, *A. westerdijkiae* grew faster in all conditions. The growth of *A. westerdijkiae* and *P. nordicum* on dry-cured ham-based media at the same temperature as this study (25 °C) is consistent with earlier findings (Vipotnik et al., 2017), who reported similar behaviour at a comparable a_w value of 0.93. Other *P. nordicum* strains have shown lower μ_m values than the values obtained for both species in the present study when cultured under the same conditions and supplemented with 10 % NaCl in the same medium (Rodríguez et al., 2014). Therefore, this parameter seems to depend on the toxigenic mould strain studied, as it has been described before (Andrade et al., 2019; Meftah et al., 2018).

The medium KCl allowed *P. nordicum* to grow faster, followed by the medium NaCl+KCl. With the lowest μ_m , medium C offered the least favourable growth conditions for this species. Additionally, *A. westerdijkiae* showed the greatest μ_m in the medium KCl, while medium C again showed the slowest growth of the mould. According to previous reports, the μ_m of ochratoxigenic *P. verrucosum* strains in YES medium increases when high KCl or NaCl concentrations are added (Andrade et al., 2019). *P. nordicum* grew faster when higher NaCl concentrations (22 % compared to 10 %) were added to the dry-cured ham-based medium, even though inter-strain differences were noted (Rodríguez et al., 2014). These results indicated that both moulds are well adapted to a rich-salt environment, growing faster as the NaCl and KCl concentrations were higher. The medium supplemented with KCl allowed the maximum growth rate, indicating a possible difference in osmoadaptation; hence, a more exhaustive evaluation was needed to know the mechanism causing the better adaptation of toxigenic moulds and its implication in mycotoxin production.

3.2. Mycotoxin production

The concentrations of OTA and OTB (the predecessor metabolite to the OTA biosynthesis in its biosynthetic pathway) are depicted in Fig. 2. The transformation from OTB into OTA is mediated by a halogenase enzyme, which introduces a chlorine atom in the final molecular structure of OTA (Gallo et al., 2017). *A. westerdijkiae* produced the highest mycotoxin amounts (Fig. 2B). These results are supported by earlier research that found that *A. westerdijkiae* produced higher levels of OTA in dry-cured ham and dry-cured ham-based medium than *P. nordicum* (Cebrián et al., 2025; Vipotnik et al., 2017). OTA values for *P. nordicum* were similar to those of Delgado et al. (2018), who used the same NaCl-supplemented culture media.

OTB levels were below those of OTA ones in all the conditions tested,

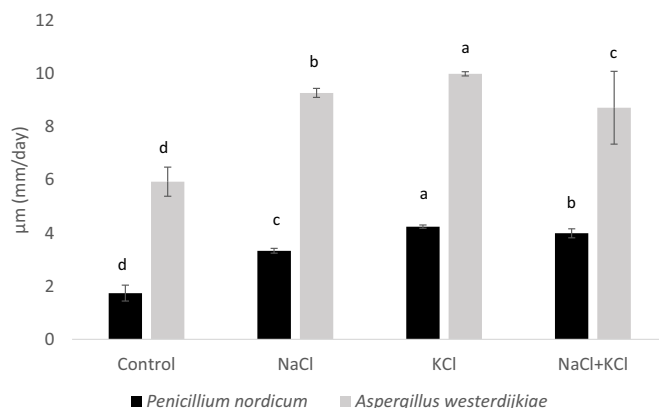


Fig. 1. Growth rate (μm) of *Penicillium nordicum* and *Aspergillus westerdijkiae* growing in different media supplemented with salts. C: Control medium; NaCl: C + 10 % (w/v) of NaCl; KCl: C + 10 % (w/v) of KCl; NaCl+KCl: C + 5 % (w/v) of NaCl and 5 % (w/v) of KCl. Different letters indicate a statistically significant difference between mean values within the same strain ($p \leq 0.05$).

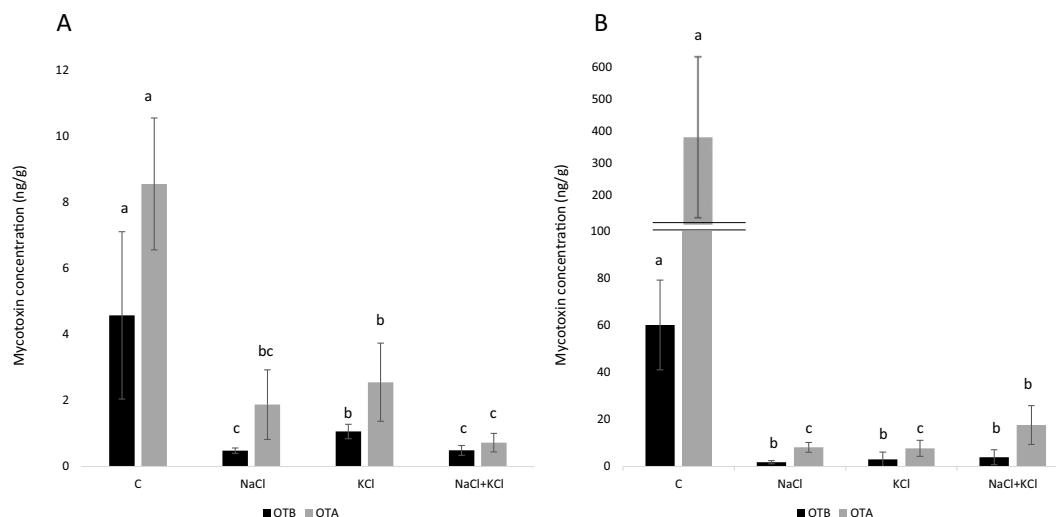


Fig. 2. Ochratoxin A and B (OTA and OTB) concentrations by *Penicillium nordicum* (A) and *Aspergillus westerdijkiae* (B) growing in different media supplemented with salts. C: Control medium; NaCl: C + 10 % (w/v) of NaCl; KCl: C + 10 % (w/v) of KCl; NaCl+KCl: C + 5 % (w/v) of NaCl and 5 % (w/v) of KCl. Different letters indicate a statistically significant difference between mean values within the same strain ($p \leq 0.05$).

suggesting the constant transformation from OTB into OTA, corroborated by the positive correlations found in both moulds ($p < 0.01$; $\rho = 0.888$ in *P. nordicum* and $\rho = 0.916$ in *A. westerdijkiae*). This also points to the halogenase enzyme was working with similar efficiency regardless of the OTB and OTA levels. Therefore, the activity of the halogenase enzyme is not influenced by the NaCl or KCl content.

Both moulds were able to produce higher OTB and OTA concentrations in medium C than in the rest of the salt-rich media (Fig. 2). Similarly, in another research, *P. nordicum* presented lower mycotoxin concentration in the same culture medium used in this work when it was supplemented with 10 % NaCl (Delgado et al., 2018). In addition, Wang et al. (2020) previously demonstrated that *P. nordicum* and *A. ochraceus* (probably *A. westerdijkiae*) grown in non-supplemented PDA presented higher OTA concentrations than in PDA supplemented with 10 % of NaCl.

Although the OTB concentration in *P. nordicum* was increased by using KCl rather than NaCl (Fig. 2A), the OTA concentration in none of the species under study was impacted by the complete substitution. A previous study about the total replacement of different concentrations of NaCl by KCl and their effect on *P. verrucosum* strains growing in YES medium showed minor variations in OTA accumulation (Andrade et al., 2019). However, the partial replacement of NaCl with KCl evaluated in this work seemed to provoke the decrease of OTA production in *P. nordicum*, even while it increased in *A. westerdijkiae*. In this sense, it has been shown that adding a small amount of NaCl (2 % w/v) to PDA medium increases the OTA production by *A. ochraceus* (probably *A. westerdijkiae*) (Wang et al., 2021), in accordance with our results. Therefore, the complete replacement of KCl with NaCl did not seem to affect the OTA production in the strains tested. However, depending on the species, partial substitution appears to cause different adaptation mechanisms in the different toxigenic moulds' species that may have an impact on the production of OTA. It is important to note that this variation could also exist at the intra-species level. Therefore, future studies should evaluate a wider range of strains to determine how representative these findings are for each species.

3.3. Glycerol determination

It has been described that different kinds of stresses can activate mycotoxin production (Álvarez et al., 2020; Rodríguez et al., 2016). The HOG pathway is a crucial stress response pathway in fungi, indispensable for survival in high osmolarity and oxidative environments by

increasing glycerol formation (Hayes et al., 2014). In addition, the disruption of this pathway has been associated with a decrease in the OTA production by *A. westerdijkiae* (Wang et al., 2023). The intracellular glycerol content produced by both ochratoxigenic moulds grown in the different culture media is shown in Table 1. *P. nordicum* produced higher glycerol concentrations than *A. westerdijkiae*. Based on the results obtained, the HOG pathway in *P. nordicum* seems to be activated to a greater extent in medium C. The HOG pathway can be cross-activated by other stressors, which would explain the induction of OTA under low salt-rich environments (Schmidt-Heydt et al., 2012). Additionally, glycerol production could be activated by another independent pathway of HOG, as it has been suggested for the yeast *Saccharomyces cerevisiae* (Chen et al., 2013). Thus, *P. nordicum* grown in medium C, not subjected to high osmotic stress levels, resulted in much higher glycerol biosynthesis activation than the mould grown under high osmotic stress circumstances. Interestingly, the glycerol quantity is strongly negatively correlated with the growth rate of *P. nordicum* ($p < 0.01$; $\rho = -0.930$), meaning that the more growth, the less glycerol is produced. The findings suggested that *P. nordicum* is highly adaptable to salt-rich substrates, and it does not need to activate glycerol biosynthesis route to survive. The fact that this strain was isolated from dry-cured ham, a rich NaCl meat substrate, may have contributed to its capacity to overcome this high salt concentration by means different from the HOG pathway (Delgado et al., 2018). This behaviour is not exclusive to the tested *P. nordicum*, since another ochratoxigenic mould, *P. verrucosum*, displayed that the magnitude of glycerol synthesis has also been inversely correlated with the degree of adaptation to hyperosmotic conditions (Schmidt-Heydt et al., 2013).

In contrast, these salts appear to activate the HOG pathway in *A. westerdijkiae* based on the glycerol content results, indicating a significant role for this mechanism in the species' adaptation to high osmotic conditions (Table 1). Notwithstanding these findings, no correlation between the OTA concentration and glycerol production was found ($p > 0.05$) in any of the moulds under investigation. Similarly, increases in NaCl concentration in the medium point to activation of HOG in *A. carbonarius* without an increase in the OTA formation (Stoll et al., 2013). Nevertheless, other metabolic routes seem to play a role in the mould's adaptation to medium C, which results in significantly higher OTA concentrations by *A. westerdijkiae* compared to those produced in salt-enriched environments, even though the growth rate is decreased.

Regarding the kind of salt, glycerol production in both moulds was

Table 1

Intracellular glycerol and free chlorine intracellularly accumulated by *Penicillium nordicum* and *Aspergillus westerdijkiae* in different culture media elaborated with dry-cured ham after 12 days at 25 °C.

Culture media ¹	Intracellular glycerol (µg/g)		Free chlorine (µg/g)	
	<i>P. nordicum</i>	<i>A. westerdijkiae</i>	<i>P. nordicum</i>	<i>A. westerdijkiae</i>
C	6424.93 ± 457.98 ^a	8.55 ± 2.00 ^c	546.97 ± 75.66 ^d	89.06 ± 25.79 ^b
NaCl	3594.04 ± 478.23 ^b	905.54 ± 79.14 ^a	5629.69 ± 654.08 ^a	2177.20 ± 279.68 ^a
KCl	2299.28 ± 79.05 ^d	762.83 ± 61.55 ^b	4313.12 ± 374.31 ^b	2960.413 ± 369.88 ^a
NaCl+KCl	2531.84 ± 215.67 ^c	710.49 ± 152.40 ^b	2679.43 ± 67.67 ^c	2244.10 ± 530.25 ^a

¹ C: Control medium without salt added; NaCl: C + 10 % (w/v) of NaCl; KCl: C + 10 % (w/v) of KCl; NaCl+KCl: C + 5 % (w/v) of NaCl and 5 % (w/v) of KCl. Statistical differences between mean values comparing the different culture media are indicated by different letters ($p \leq 0.05$).

more significantly triggered by NaCl. Thus, a potential replacement of NaCl with KCl would suppress the HOG pathway rather than increase its utilisation.

3.4. Free chlorine determination

The free chlorine amount has been analysed due to previous studies have suggested that high NaCl environments provoke the elimination of Cl⁻ through the OTA molecule to cope with this stress in *Penicillium* species (Schmidt-Heydt et al., 2012). The concentration of free chlorine produced by both moulds was higher in the three media with salt supplementation (NaCl, KCl, and NaCl+KCl) compared to medium C, highlighting the value obtained for *P. nordicum* in the presence of NaCl (Table 1). However, no differences were detected in *A. westerdijkiae*, regardless of the type of salt added. These results support the idea of dissimilar mechanisms employed by these two mould species to overcome the osmotic stress, although both triggered a clear activation of chlorine accumulation under high osmotic pressure. This increase was previously reported by other authors in *P. nordicum* (Schmidt-Heydt et al., 2012), although this parameter in *A. westerdijkiae* remained unknown so far. Since OTA can be utilized to create such a mycotoxin from OTB by adding the Cl⁻, the low quantities of free chlorine in medium C for both toxigenic strains may be the result of increased OTA production (Fig. 2) in addition to lower free chlorine availability. This is in accordance with the up-regulation of the gene that codified a halogenase, responsible for the introduction of the chlorine atom in the OTA molecule (Ferrara et al., 2016) by *A. carbonarius* in a medium with NaCl (Wang et al., 2020). However, in the present study, the OTA and free chlorine accumulation were not correlated in any tested mould and condition. Therefore, to deal with the osmotic stress, both moulds would be excreting chlorine atoms through the OTA molecules and free chlorine. In this sense, *P. nordicum* and *A. westerdijkiae* display a common shared feature about chlorine excretion, regardless of the previous physiologic differences reported.

4. Conclusions

This study shows that the growth and OTA production of *A. westerdijkiae* and *P. nordicum* are affected differently when KCl is substituted for NaCl in dry-cured ham-based media. High adaptation to salt-rich conditions was demonstrated by the faster development of both moulds in salt-supplemented media, with *A. westerdijkiae* exhibiting overall higher growth and OTA production. Medium C, which was not supplemented, had the highest OTA levels. Glycerol content and free chlorine production varied by salt type and species, indicating distinct osmoadaptation mechanisms. *A. westerdijkiae* triggered the HOG pathway, whereas *P. nordicum* seemed not to use glycerol biosynthesis route under salt stress conditions. Chlorine excretion occurred via OTA and free chlorine in both species, though no direct correlation with OTA levels was found. Total NaCl replacement by KCl did not significantly alter OTA synthesis, suggesting feasibility for sodium reduction in dry-cured ham. However, partial NaCl substitution may risk elevated OTA production by *A. westerdijkiae*. These findings highlight species-specific

responses to NaCl/KCl substitution and underscore the need for tailored mitigation strategies to ensure food safety in low-sodium dry-cured ham.

CRedit authorship contribution statement

Micaela Álvarez: Writing – original draft, Investigation, Formal analysis. **Josué Delgado:** Writing – review & editing, Methodology, Investigation. **Alicia Rodríguez:** Writing – review & editing, Supervision, Methodology, Funding acquisition, Conceptualization.

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Declaration of competing interest

The author is an Editorial Board Member/Editor-in-Chief/Associate Editor/Guest Editor for this journal and was not involved in the editorial review or the decision to publish this article.

Data availability

Data will be made available on request.

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