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Unravelling the modes of action of autochthonous staphylococci against *Aspergillus westerdijkiae* and the antiochratoxigenic effect in dry-cured ham

Eva Cebrián, Mar Rodríguez, Micaela Álvarez, Josué Delgado *, Félix Núñez

Higiene y Seguridad Alimentaria, Instituto de Investigación de Carne y Productos Cárnicos (IProCar), Facultad de Veterinaria, Universidad de Extremadura, Avda. de las Ciencias s/n, 10003, Cáceres, Spain

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ABSTRACT

Aspergillus westerdijkiae is well known for its capacity to produce high amounts of ochratoxin A (OTA) in dry-cured ham. Some autochthonous microorganisms, such as *Staphylococcus xylosus* FHSCC Sx8 and *Staphylococcus equorum* FHSCC Se31 have antifungal effect and can be used as biocontrol agents (BCAs) to reduce the OTA contamination. The aims of this work were to reveal their modes of action against *A. westerdijkiae* MUM 16.142 in a dry-cured ham model system and validate the antifungal effect in dry-cured ham. As well as to assess the effect of live cells, dead cells, and cell-free broth; the pattern of nutrient use, the niche overlap index (NOI), interactions by dual culture assays, the antifungal activity of volatile compounds, and their effect on the fungal proteome. Both cocci in the dual-culture assay were able to decrease the *A. westerdijkiae* growth, but only *S. xylosus* reduced the OTA production at 20 °C. Moreover, changes in the abundance of proteins related to cell wall integrity, secondary metabolite production and carbohydrate metabolism were observed. Furthermore, the anti-*A. westerdijkiae* effect of *S. xylosus* was validated in dry-cured ham since this coccus was able to reduce the OTA production in this meat product.

1. Introduction

The mould growth on the surface of the dry-cured ham during ripening is common due to their adaptation to these environmental conditions (Battilani et al., 2007; Núñez, Rodríguez, Bermúdez, Córdoba, & Asensio, 1996). Although fungal growth positively contributes to the sensory characteristics of the final product (Martín, Córdoba, Aranda, Córdoba, & Asensio, 2006; Martín, Córdoba, Núñez, Benito, & Asensio, 2004), some moulds have the capacity to produce mycotoxins. The most common mycotoxin found in dry-cured ham is the ochratoxin A (OTA) (Bertuzzi, Gualla, Morlacchini, & Pietri, 2013). Although *Penicillium nordicum* is considered the main producer (Battilani et al., 2007; Rodríguez, Rodríguez, Martín, Delgado, & Córdoba, 2012), *Aspergillus westerdijkiae* can also generate a high amount of OTA in dry-cured ham (Rodrigues et al., 2019; Vipotnik, Rodríguez, & Rodrigues, 2017). OTA has a negative impact on consumers' health, with nephrotoxic, immunosuppressive, genotoxic, carcinogenic, teratogenic and neurotoxic effects (Klingelhöfer et al., 2020). In addition, it had been included into group 2B as possibly carcinogenic to humans (IARC, 1993), although it is likely to be reclassified in the group 2A (Ostry, Malir, Toman, & Grosse, 2017).

In view of this hazard, it is crucial to implement strategies in the meat industry to control the growth of toxigenic moulds and OTA production. The application of biocontrol agents (BCAs) in dry-cured meat products seems to be the most promising strategy because they do not cause negative alterations, such as certain physical and chemical methods (Asensio, Núñez, Delgado, & Bermúdez, 2014). In this respect, the efficacy of certain yeasts (Núñez et al., 2015; Peromingo, Núñez, Rodríguez, Alía, & Andrade, 2018), non-toxigenic moulds (Cebrián, Rodríguez, Peromingo, Bermúdez, & Núñez, 2019; Delgado, Peromingo, Rodríguez, & Rodríguez, 2019; Álvarez, Núñez, et al., 2021), lactic acid bacteria (Álvarez, Rodríguez, Peromingo, Núñez, & Rodríguez, 2019) and gram-positive catalase-positive cocci (GCC+) (Cebrián et al., 2020; Cebrián, Núñez, Álvarez, Roncero, & Rodríguez, 2022) has been demonstrated to decrease the growth of mycotoxin-producing moulds and to reduce the OTA production in different meat products. GCC+ are one of the most predominant microbial groups during the ripening of dry-cured ham (Rodríguez, Núñez, Córdoba, Bermúdez, & Asensio, 1996). Moreover, some of them have been described as potential BCAs against toxigenic moulds in several foods (Alijani, Amini, Ashengroph, & Bahramnejad, 2019; Cebrián et al., 2020; Gong, Sun, Zhao, Liao, & Zhang, 2020). In fact, *Staphylococcus xylosus* Sx8 and *Staphylococcus*

* Corresponding author.
E-mail address: jdperon@unex.es (J. Delgado).

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equorum Se31 are effective in delaying the growth of mycotoxin-producing moulds and significantly reduce the production of OTA, cyclopiazonic acid (CPA) and aflatoxins (Cebrián et al., 2020) in meat substrates. However, their modes of action are not still known.

The elucidation of the modes of action of BCAs is essential to optimise their application during the ripening. The generation of volatile compounds has been related to the antifungal effect of some staphylococci. In this respect, *Staphylococcus sciuri* inhibited the growth of *Colletotrichum nymphaeae* due to the volatile compounds (Alijani et al., 2019), and the mycelial proliferation of *Aspergillus flavus* is limited by the 3,3-dimethyl-1,2-epoxybutane produced by *Staphylococcus saprophyticus* (Gong et al., 2020). Additionally, competition for space and nutrients, proteins production, repression of genes involved in the OTA biosynthetic pathway or degradation or absorption of mycotoxins have been also described as modes of action related to the antifungal activity of different BCAs in dry-cured meat products (Andrade, Thorsen, Rodríguez, Córdoba, & Jespersen, 2014; Cebrián et al., 2019; Crowley, Mahony, & van Sinderen, 2013; Dalié, Deschamps, & Richard-Forget, 2010; Delgado, Owens, Doyle, Asensio, & Núñez, 2015; Gil-Serna, Patiño, Cortés, González-Jaén, & Vázquez, 2011; Peromingo et al., 2018; Álvarez, Núñez, et al., 2021).

On the other hand, comparative proteomic analysis is a useful tool to elucidate the mode of action of these microorganisms. Some studies have demonstrated that some BCAs can interfere in proteins related to the cell wall integrity (CWI) and/or to the secondary metabolites biosynthesis (Delgado, Núñez, Asensio, & Owens, 2019; Álvarez, Delgado, Núñez, Cebrián, & Andrade, 2021; Álvarez, Delgado, Núñez, Roncero, & Andrade, 2022; Álvarez, Núñez, Delgado, Andrade, & Rodrigues, 2022). Thus, its application to this purpose is of utmost interest to reveal the main pathways affected in *A. westerdijkiae* by the BCAs.

Therefore, the aims of this work were: a) to reveal the effect of live cells, dead cells and extracellular compounds, including volatile compounds of *S. xylosus* Sx8 and *S. equorum* Se31 on *A. westerdijkiae* and its OTA production; b) to evaluate the nutrient and space competition between these cocci and the toxigenic mould; c) to assess the impact of these bacteria on *A. westerdijkiae* in a dry-cured ham-based medium at environmental conditions usually found during the ripening of dry-cured ham, and d) evaluating the mould proteome to unveil the possible modes of action involved in the antifungal effect. Finally, e) this ability was further validated on dry-cured ham.

2. Materials and methods

2.1. Microorganisms and inocula preparation

The OTA-producer *A. westerdijkiae* MUM 16.142 belonging to the Micoteca da Universidade do Minho (Braga, Portugal) and two staphylococci, *S. xylosus* FHSCC Sx8 and *S. equorum* FHSCC Se31 from the Culture Collection of Food Hygiene and Safety of the University of Extremadura (Cáceres, Spain) were used. All three microorganisms had been isolated from dry-cured hams (Rodríguez et al., 1994; Vipotnik et al., 2017).

The mould was grown on Potato Dextrose agar (PDA, Conda Pronadisa, Spain) for 7 days at 25 °C. The spores were collected using 3 mL of phosphate buffer saline (PBS) and scraping the surface with a sterile glass rod. The spores were counted in a Thoma chamber Blaubrand ® (Brand, Germany) and adjusted to 10^6 spores/mL to be used as inoculum.

The inocula of both staphylococci for dual-culture assay, carbon sources utilisation and volatile compounds assay were obtained by growing them on brain heart infusion broth (BHI, Condalab, Spain) at 30 °C for 48 h. For live cells, dead cells and cell-free broth assays the cocci were grown in meat extract broth (MEB, Scharlab, Spain). After 48 h, cultures were centrifuged, and the pellet was resuspended in PBS. Then, counts were obtained by plating on Mannitol Salt Agar (MSA, Condalab). At the end, the cell suspensions were adjusted to 10^8 cfu/mL and used as inocula in all assays made in dry-cured ham-based agar (DHA). For the study in dry-cured ham, the cell suspensions were adjusted to 10^6 cfu/mL to be used as inocula.

2.2. Effect of live cells, dead cells, and extracellular compounds of staphylococci on A. westerdijkiae growth and OTA production

This study was carried out following the methodology previously described by Meftah Abid, Dias, and Rodrigues (2020) in DHA prepared as described by Cebrián et al. (2020) with a final water activity (a_w) value of 0.95 as that reached in the post-salting phase, in which GCC+ initiate their exponential growth. The use of a meat model allows the study of the antifungal mode of action of microorganisms under controlled conditions (Crowther, Boddy, & Maynard, 2018). The cultures of the two cocci in MEB was divided into 2 portions. The first portion was centrifuged at 2377×*g* for 5 min in a centrifuge model 5430 (Eppendorf AG, Germany) to obtain the pellet and the supernatant. The pellet resuspended in sterile PBS was used for the live cell assay. The supernatant filtered through a 0.22 μm pore size nylon membrane (RephiLe Bioscience, USA) was used for the cell-free assay.

The second portion was used for the dead cell assay. For this purpose, it was autoclaved at 121 °C for 20 min and then centrifuged at 2377×*g* for 5 min in a centrifuge model 5430 (Eppendorf AG). The pellet resuspended in sterile PBS was used as inoculum.

On the other hand, to test the possible extracellular antifungal activity, each of the cell-free extracts were separated by ultrafiltration using Vivaspin 2 centrifugal concentrators (MWCO 2 kDa, Sartorius Stedim Lab Lid., UK) obtaining two parts: a concentrated portion containing the compounds with a MW $\geq$ 2 kDa and a non-concentrated containing the rest components of the extract with a MW $<$ 2 kDa.

To carry out the assays, 50 μL of each suspension were spread onto the surface of the DHA in 5 cm Petri dishes. Then, 2 μL of the mould spore's suspension were inoculated at a single point in the centre of all plates. The control batch was inoculated only with the mould. All cultures were incubated for 14 days at 15 °C and 20 °C, temperatures usually found during the ripening of dry-cured ham (Rodríguez et al., 1994). The experiment was performed in triplicates.

The diameter of *A. westerdijkiae* colonies in the presence or absence of each fraction was measured every two days in two perpendicular directions. The inhibitory activity (IA) was expressed in percentage as follows: IA (%) = [(C-T)/C] x 100, where C was the average diameter of mould colonies growing in the absence of any added extract from *S. xylosus* or *S. equorum* and T was the average diameter of *A. westerdijkiae* colonies in batches inoculated with live cells, dead cells, or cell-free extracts of staphylococci.

In addition, OTA was extracted, detected, and quantified as described in Section 2.7 after 14 days of incubation.

2.3. Carbon sources (CS) pattern

For CS utilisation patterns sterile 96-well microtiter plates, resazurin sodium salt solution (RSS; Sigma-Aldrich, USA), and dissolutions of 20 CSs selected among the main constituents of dry-cured meat products were used including fructose, glucose, lactose, sucrose, ribose, glycine, alanine, arginine, asparagine, cysteine, phenylalanine, glutamine, histidine, isoleucine, lysine, methionine, proline, valine, serine and maltose (Sigma-Aldrich). The RSS solution was prepared by adding 2 g/L of RSS to PBS modified with 50 g/L NaCl (minimal media), and 15 μL were poured into each well. Then, 170 μL of each CS solution, filtered through a 0.22 μm-pore size membrane (RephiLe Bioscience), were added to the minimal media to reach a final concentration of 20 g/L. Next, they were inoculated with 15 μL of the respective microorganism (10^8 cfu/mL of each GCC+ or 10^6 spores/mL of *A. westerdijkiae*). The assay was run both at 15 and 20 °C for 7 days in triplicate wells, using separate plates of each species.

The metabolization of each CS by each microorganism was visually checked since resazurin, a nonfluorescent blue dye, is converted by reduction into the pink-coloured fluorescent resorufin, revealing the viability of the microorganism (Monteiro et al., 2012). In addition, every two days, the data were quantified by measuring fluorescence (excitation 570 nm, emission 615 nm) following the methodology described by Monteiro et al. (2012).

The niche overlap index (NOI) was estimated for each staphylococci against *A. westerdijkiae* and vice versa at both tested temperatures based on the number of CSs used for each isolate according to the following formula (Wilson & Lindow, 1994).

$$NOI = \frac{Number\ of\ CSs\ in\ common\ between\ two\ microorganism}{Number\ of\ CSs\ utilised\ by\ the\ tested\ microorganism}$$

The NOI values obtained are between 0 and 1. NOI values > 0.90 indicate coexistence in the same nutritional niche and values < 0.90 indicate the occupation of different niches.

2.4. Antifungal effect of volatile compounds produced by autochthonous staphylococci

The effect of volatile compounds produced by *S. xylosus* and *S. equorum* was examined using the "mouth to mouth" method (Núñez et al., 2015), resulting in a double-dish system (DDS). This assay was also performed on DHA to homogenize the volatile compounds of the matrix and prevent any external microorganism's volatile compound. On the upper plate 50 μL of a 10^8 cfu/mL suspension of each coccus were spread. The lower plate was inoculated with 2 μL of a 10^6 spores/mL of *A. westerdijkiae* in a single point in the centre of each plate. After drying, the lids of the petri dishes were removed, and the plates were faced each other and sealed with parafilm™ (Dilab, Ecuador). The plates were incubated at 15 and 20 °C for 14 days. As negative control, a DDS without any inoculation, and as positive control without the inoculation of cocci in the upper plate were performed. To study the inhibitory activity of GCC+, the diameter of the colonies of *A. westerdijkiae* was daily measured in two perpendicular directions and the IA was calculated as described in Section 2.2.

In addition, the production of OTA by *A. westerdijkiae* was analysed in absence and presence of both staphylococci as described in Section 2.7.

2.5. Dual-culture assay

To study the interaction of autochthonous staphylococci with the ochratoxigenic mould, a dual-culture assay was performed on DHA medium following the methodology previously described by Magan and Lacey (1984). *A. westerdijkiae* was inoculated at a single point 20 mm apart from the corresponding BCA inoculated on a streak by adapting the methodology described by Álvarez, Núñez, et al. (2021). The control batch was inoculated only with *A. westerdijkiae*. All plates were incubated for 14 days at 15 and 20 °C. The experiment was performed in triplicate.

The diameter of *A. westerdijkiae* colonies in the presence or absence of each GCC+ was measured every two days in two perpendicular directions. The IA was calculated as described in Section 2.2.

After 14 days, the interaction of *A. westerdijkiae* with *S. xylosus* and *S. equorum* were macroscopically observed and the Index of Dominance (I_D) was determined using the classification system and the numerically scored provided by Magan and Lacey (1984). The OTA amount was analysed as described in Section 2.7.

2.6. Effect of S. xylosus on A. westerdijkiae growth and OTA production in dry-cured ham

This study was carried out on dry-cured ham pieces of approximately 7 × 5 × 1.5 cm. To reduce microbial contamination, the pieces were previously sanitized by immersion in pure ethanol for 30 s and then exposed to ultraviolet light for 15 min on each side.

Two different batches were prepared: the control batch, only inoculated with *A. westerdijkiae* and the other one, inoculated with *A. westerdijkiae* and *S. xylosus.* Fifty μL of each stock solutions prepared for each microorganism, as described in Section 2.1., were inoculated on the surface of each portion using a Drigalsky spreader and kept drying for a 5 min between every inoculation. After that, the inoculated pieces were incubated at 20 °C for 14 days in plastic containers previously sterilized. To simulate the environmental humidity during the ripening of dry-cured ham, 250 mL of sterile oversaturated KNO_3 solution were deposited at the bottom of the containers to reach a relative humidity of 94%, equivalent to 0.94 a_w, very close to that of DHA. The experiment was carried out in quintupled.

To assess the mould and GCC+ growth, at the end of incubation time, 5 g of each dry-cured ham portion was homogenized with 45 mL of 0.1% peptone water in a filter bag using a Stomacher. After performing decimal dilutions, the mould growth was determined by plating on Rose Bengal Agar (RB, Condalab) and incubating at 25 °C for 7 days. The GCC+ growth was studied by plating in MSA (Condalab) at 30 °C for 48h.

In addition, the production of OTA by *A. westerdijkiae* was analysed in both batches as described in Section 2.7.

2.7. OTA extraction and quantification

After incubation of the cultures described in Sections 2.2, 2.4, and 2.5, 1 g of agar including mycelium was collected from the closest area to the cocci. For the assay described in section 2.6, 5 g of each dry-cured ham portions was collected. OTA was extracted using the QuEChERS methodology described by Delgado, da Cruz Cabral, Rodríguez, and Rodríguez (2018). For DHA samples, the following procedure was followed: briefly, the samples were mixed with 2 mL of water acidified with acetic acid 0.1% (v/v; Fisher Scientific, USA) and 2 mL of acetonitrile acidified with acetic acid 0.1% (v/v). Then, 0.4 g of NaCl (Fisher Scientific) and 1.6 g of anhydrous $MgSO_4$ (Scharlab, S.L) were added to the tubes and immediately shaken. Next, the samples were centrifuged at 2630×*g* for 5 min (Orto Alresa digtor 21R, Spain) and 1 mL of supernatant was collected. Finally, the samples were diluted to 1:1 with water and filtered through a 0.22 μm pore-size nylon membrane (RephiLe Bioscience). For dry-cured ham samples, the process was the same but multiplying the added quantities by three.

OTA was analysed using a Q Exactive Plus mass spectrometer (Thermo Fisher Scientific) following the method described by Cebrián et al. (2020). A Thermo Fisher Accucore Aq C18 of 150 × 2.1 mm, 2.6 μm particle size column was used. The mobiles phases A (H_2O 0.1% formic acid) and B (acetonitrile 0.1% formic acid) with a flow rate of 0.3 mL/min was used. Injection volume was set in 10 μL. OTA was eluted by 6.71 ± 0.1 min. The calibration curve of OTA was prepared using different levels from 0.01 to 100 ng/mL and revealed a linear relationship ($R^2 \geq 0.99$) for every working range.

2.8. Comparative proteomic analysis

After 12 days of incubation at 20 °C two comparisons were made. The first one compared the control batch with the batch inoculated with *S. xylosus* and the second one compared the batch inoculated with *S. equorum* and the batch inoculated with *S. xylosus* in order to observe the proteomic changes happening when OTA was reduced by the effect of one coccus and when it was not by the other one, as described in Section 3.4.

The experiment of dual-culture assay described in Section 2.5 at 20 °C was repeated in quintuplicate for performing a further comparative proteomics study, since the presence of *S. xylosus* caused reduction in the amount of OTA but *S. equorum* did not. For this, after 12 days of incubation, half of fungal mycelia (n = 5) were removed by scraping,

and were immediately flash frozen in liquid nitrogen and stored at −80 °C until extraction, following the methodology described by Álvarez, Delgado, Núñez, Cebrián, and Andrade (2021). Lysis buffer pH 7.5 was added to the samples together with pepstatin A (1 μg/mL) (Sigma-Aldrich) and PMSF (1 mM) (Sigma-Aldrich) and then lysed by sonication. The lysates clarified by centrifugation were partially run on SDS-PAGE to concentrate all the proteins from each sample into a single band.

Each of the bands were cut and reduced with dithiothreitol (Promega, Madison, USA) and alkylated with iodoacetamide (Promega). Finally, the proteins were digested with a combined solution of ProteaseMAX (Promega) and sequencing-grade trypsin (Promega) for 1 h at 50 °C, according to manufacturer instructions.

A total of 2 μg of the resulting peptides were analysed using a Q-Exactive mass spectrometer coupled to a Dionex Ultimate 3000 RSLC nano (Thermo Scientific) following the methodology described by Delgado, Núñez, Asensio, and Owens (2019). Label-free comparative proteome abundance and data analysis were performed using MaxQuant software (v. 1.6.15.0; https://www.maxquant.org/download_asset/maxquant/la test), with Perseus (v. 1.6.14.0) applied to organise the data and conduct statistical analyses. An annotated database of *A. westerdijkiae* proteins was created for comparison with the peptides obtained in a previous study (Álvarez, Núñez, et al., 2022).

The maximum peptide/protein false discovery rates (FDR) were set to 1% based on comparison to a reverse database. The label-free quantitative algorithm (LFQ) was used to generate normalised spectral intensities and infer relative protein abundance (Luber et al., 2010). Proteins that matched with a contaminant database or the reverse database were removed, and proteins were only retained in final analysis if detected in at least three biological replicates from at least one treatment. Quantitative analysis was performed using a *t*-test to compare treatments with the control ($p < 0.05$). Qualitative analysis was also performed to detect proteins found in at least three biological replicates of a particular treatment but undetectable in the compared treatment. For enrichment analysis, the proteins were evaluated trough ClueGO (v. 2.5.6) (Bindea et al., 2009). To define term-term interrelations and functional groups based on shared genes between the terms, the Kappa score was stablished at 0.4. Three GO terms and 4% of genes covered were set as the minimum required to be selected. The *p*-value was corrected by Bonferroni step down and set as $p \leq 0.05$.

2.9. Statistical analysis

All data were analysed using IBM SPSS Statistics v22.0 (IBM Corporation, USA). Data normality test was performed by Shapiro-Wilks. All the variables followed a normal distribution except growth and OTA data in dual-culture assay, and in the growth in volatile compounds assay. To determine significant differences, ANOVA analysis was carried out for parametric data for normally distributed data. Dunnet's T3 test was applied to compare the treated samples with the control batches. For the variables that failed in the normally test, non-parametric data analysis was performed using the Kruskal-Wallis test and then, the U Mann-Whitney test was applied to detect differences between batches and to compare the median values obtained respectively.

3. Results

3.1. Antifungal effect of live cells, dead cells, and extracellular compounds of staphylococci

3.1.1. Effect on A. westerdijkiae growth

The live cells of *S. xylosus* and *S. equorum* caused the total inhibition of ochratoxigenic *A. westerdijkiae* growth at both 15 and 20 °C. Neither the dead cells nor the extracellular extract from both staphylococci caused a significant reduction of *A. westerdijkiae* at either 15 or 20 °C (Table 1).

Table 1
Inhibitory effect of *Staphylococcus xylosus* FHSCC Sx8 (Sx) and *Staphylococcus equorum* FHSCC Se31 (Se) on the growth of *Aspergillus westerdijkiae* MUM 16.142 (Aw) after 14 days at 15 and 20 °C on dry-cured ham-based agar with the different treatments.

Treatments	% Inhibition of *Aspergillus westerdijkiae* growth			
	Aw-Sx		Aw-Se	
	15 °C	20 °C	15 °C	20 °C
Live cells	100*	100*	100*	100*
Dead cells	1.27	0.00	0.12	0.00
Cell-free broth	6.93	0.00	3.58	0.00
Extracellular compounds >2 kDa	3.00	0.00	0.69	2.92
Extracellular compounds <2 kDa	3.00	−0.66	−0.46	2.59
Dual culture	16.28*	12.00*	15.51*	12.00*
Volatile compounds	6.11*	0.00	9.47*	0.00

*Statistically significant differences with respect to the control ($p \leq 0.05$).

3.1.2. Effect on OTA production

In the batches co-inoculated with the live cells of *S. xylosus* and *S. equorum*, the OTA production by *A. westerdijkiae* was not analysed since no fungal growth was observed. In the rest of the treatments, no significant changes in the OTA amount were obtained with respect to the non-treated *A. westerdijkiae* (data not shown).

3.2. Patterns in utilisation of carbon sources

The total of CSs utilised by *A. westerdijkiae*, *S. xylosus* and *S. equorum* at 15 and 20 °C are shown in Fig. 1. The highest number of CSs (18) was used by *A. westerdijkiae* at both temperatures. As regards the BCAs, both *S. xylosus* and *S. equorum* metabolised more CSs at 15 °C than at 20 °C.

The CS utilisation patterns were used to determine the NOI values of BCAs in relation to *A. westerdijkiae* and their ecological similarity. In this respect, *A. westerdijkiae* was able to dominate both staphylococci at the two studied temperatures. Nevertheless, the ochratoxigenic mould and *S. equorum* coexisted at 15 °C (Fig. 2).

3.3. Antifungal effect of staphylococcal volatile compounds

3.3.1. Effect on fungal growth

The volatile compounds generated by both *S. xylosus* and *S. equorum* on dry-cured ham-based agar caused a significant decrease in the growth of *A. westerdijkiae* at 15 °C, but not at 20 °C after 14 days of incubation (Table 1).

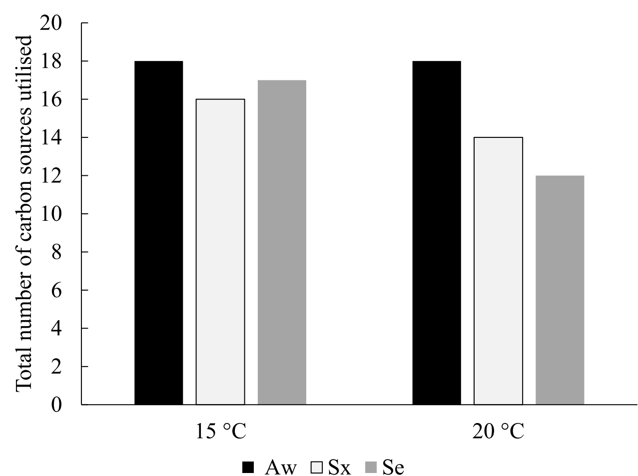

Fig. 1. Total number of carbon sources used by *Aspergillus westerdijkiae* MUM 16.142 (Aw), *Staphylococcus xylosus* FHSCC Sx8 (Sx) and *Staphylococcus equorum* FHSCC Se31 (Se) at 15 and 20 °C.

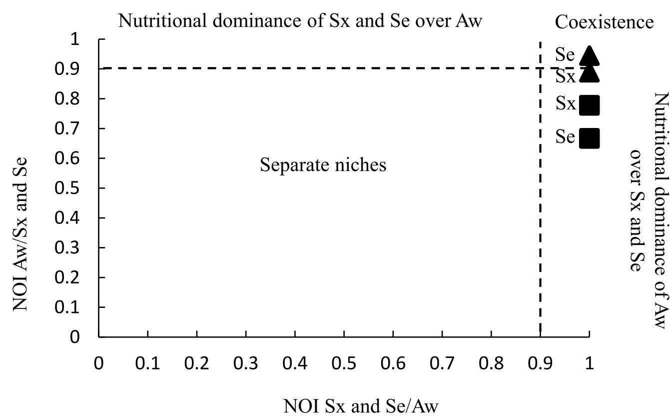

Fig. 2. Niches overlap indices (NOIs) between *Aspergillus westerdijkiae* MUM 16.142 (Aw) and *Staphylococcus xylosus* FHSCC Sx8 (Sx) and *Staphylococcus equorum* FHSCC Se31 (Se) at 15 (▲), and 20 °C (■).

3.3.2. Effect on OTA production

The volatile compounds produced from staphylococci did not have any impact on the amount of OTA produced by *A. westerdijkiae* at the end of the incubation (data not shown), even at 15 °C where a small reduction of fungal growth was observed.

3.4. Antifungal effect of staphylococci in dual cultures on dry-cured ham-based agar

3.4.1. Effect on fungal growth

Both BCAs significantly decreased the growth of *A. westerdijkiae* at 15 and 20 °C at the end of incubation (Table 1). *S. xylosus* and *S. equorum* inhibited the growth of *A. westerdijkiae* by contact at the two studied temperatures (Fig. 3), and the macroscopic interactions between the mould and each staphylococcus were recorded as the total I_D score (Table 2)

3.4.2. Effect on OTA production

The amount of OTA was significantly reduced ($p \leq 0.05$) when *A. westerdijkiae* was inoculated with *S. xylosus* at 20 °C after 14 days of incubation. However, neither *S. xylosus* at 15 °C, nor *S. equorum* at both temperatures significantly impacted on the OTA amount produced (Fig. 4).

Table 2
Interaction scores and Index of Dominance (I_D) between *Aspergillus westerdijkiae* MUM 16.142 and *Staphylococcus xylosus* FHSCC Sx8 (Aw-Sx) and *Staphylococcus equorum* FHSCC Se31 (Aw-Se) at 15 and 20 °C after 14 days of incubation on dry-cured ham-based agar.

	Interaction scores		I_D
	15 °C	20 °C	
Aw-Sx	4	4	8
Aw-Se	4	4	8

Interaction scores: 0: no interaction; 1: Mutual intermingling; 2: Mutual inhibition on contact or space between colonies small (<2 mm); 3: Mutual inhibition at a distance (>2 mm); 4: Inhibition of the mould on contact. 5: Inhibition of the mould at a distance. Adapted from Magan and Lacey (1984).

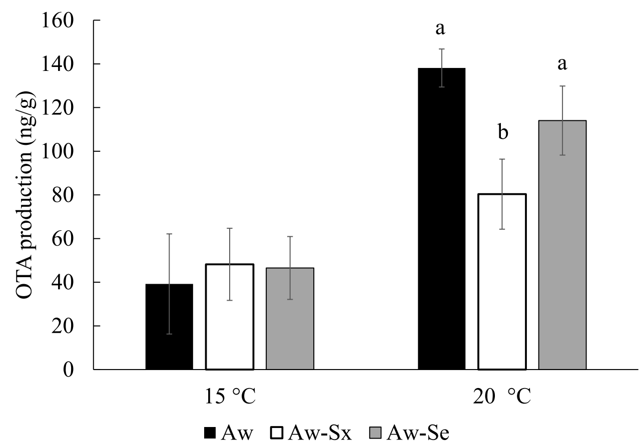

Fig. 4. Effect of *Staphylococcus xylosus* FHSCC Sx8 (Aw-Sx) and *Staphylococcus equorum* FHSCC Se31 (Aw-Se) on OTA production by *Aspergillus westerdijkiae* MUM 16.142 (Aw, untreated control) after 14 days at 15 and 20 °C on dry-cured ham-based agar. * Values of OTA concentration within the same temperature with significant differences are denoted with different letters ($p \leq 0.05$).

3.4.3. Comparative proteomic analyses

Given that *S. xylosus* caused a significant reduction in OTA production by *A. westerdijkiae* at 20 °C, but this effect was not observed on

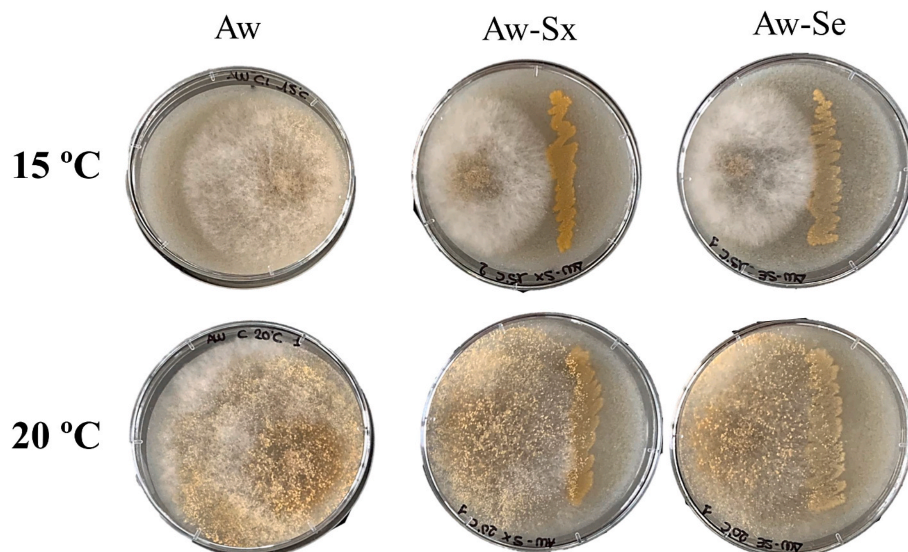

Fig. 3. Interaction between *Aspergillus westerdijkiae* MUM 16.142 (Aw, untreated control) with *Staphylococcus xylosus* FHSCC Sx8 (Aw-Sx) and *Staphylococcus equorum* FHSCC Se31 (Aw-Se) at 15 and 20 °C after 14 days of incubation on dry-cured ham-based agar.

S. equorum, two comparative proteomic analyses were performed to clarify the anti-ocratoxigenic mode of action of *S. xylosus*. Firstly, *A. westerdijkiae* untreated control (Aw) *versus* this mould in dual culture with *S. xylosus* (Aw-Sx, which provoked OTA inhibition) resulting in the comparison Aw *vs* Aw-Sx. And secondly, the batches inoculated with each coccus: not OTA inhibiting coccus (Aw-Se) *versus* OTA inhibiting coccus (Aw-Sx) resulting in Aw-Se *vs* Aw-Sx, were analysed, in both cases after 12 days of incubation at 20 °C. The LFQ proteomic analyses for the three batches resulted in 1270 total proteins identified. Quantitative ($p < 0.05$) and qualitative (detected uniquely in one of the conditions) variations in protein amounts were observed in each treatment, compared to the control or to the batch inoculated with *S. equorum*. The number of proteins altered in abundance are displayed in Table 3.

In the comparative Aw *vs* Aw-Sx, the LFQ analysis identified a total of 1269 proteins (Supplementary Table S1). For this comparation the gene ontology enrichment analysis classified the proteins in groups which were subdivided in more specific terms. ClueGo divided the increase proteins identified in 45 metabolic routes or terms, whereas the decreased proteins were included in 58 terms. Most of the terms found increased (48.89%) were incorporated in the "proteasome core complex, alpha-subunit complex" group, followed by the "organonitrogen compound catabolic process" group (28.89%) (Fig. S1). The terms for proteins found in lower relative abundance were mainly grouped in "cellular amide metabolic process" (55.17%) and "carboxylic acid metabolic process" (20.69%) groups (Fig. S2).

Regarding the proteins related to the CWI pathway, some of them such as cofilin, tubulin alpha chain, cell surface spherulin 4 family protein, glutamine-fructose-6 phosphate transaminase and septin were found in lower abundance by the effect of *S. xylosus* (Table 3). However, *S. xylosus* also seems to enhance the abundance of other proteins related to this pathway such as glycosidase. In addition, BAR adaptor protein RVS161 putative was only detected in the staphylococci co-inoculated batch (Table 4).

Furthermore, changes in the abundance of *A. westerdijkiae* proteins related to secondary metabolite biosynthesis were also observed. In this regard, adenylyl cyclase-associated protein 1, 4-hydroxyphenylpyruvate dioxygenase and acetyl-CoA carboxylase, putative were decreased in abundance whereas homogentisate 1,2-dioxygenase was increased in the presence of *S. xylosus* (Table 4).

In addition, two proteins related to the oxidative stress response such as superoxide dismutase and glutathione-S-transferase omega, putative were altered in abundance by *S. xylosus*.

Finally, *S. xylosus* also caused variations in the abundance of proteins involved in stress response and carbohydrate metabolism. In the latter group, the abundance of 6 out of 10 proteins was decreased.

On the other hand, comparative proteomics among the batch co-inoculated with *S. equorum* (Aw-Se, where OTA is not reduced, Fig. 4) *versus* the batch co-inoculated with *S. xylosus* (Aw-Sx, being OTA reduced, Fig. 4) was carried out to detect the changes that underlie the OTA repression. The LFQ analysis identified a total of 1233 proteins for this comparation (Supplementary Table S2). The number of proteins altered in abundance are displayed in Table 3.

Considering the small amount of data obtained in this comparison, the ClueGo analysis was not very effective as its results were only obtained for the increased proteins in the presence of *S. xylosus*. These proteins were included in 2 terms being the 50% of them classified per groups in "alpha-amino acid biosynthetic process" and the other 50% in "metallopeptidase activity" (Fig. S3).

Alcohol dehydrogenase was the only CWI pathway-related protein altered in abundance in the presence of *S. xylosus* (Table 5). On the other

Table 3
Number of proteins altered in abundance in *Aspergillus westerdijkiae* MUM 16.142 proteome after 12 days at 20 °C on dry-cured ham-based agar. Aw: control batch inoculated with *A. westerdijkiae*; Aw-Sx batch co-inoculated with *A. westerdijkiae* and *Staphylococcus xylosus* FHSCC Sx8; Aw-Se batch co-inoculated with *A. westerdijkiae* and *Staphylococcus equorum* FHSCC Se31.

Comparative analysis	Proteins reduced in abundance	Proteins increased in abundance	Proteins only detected in Aw-Sx	Proteins only detected in Aw
Aw- *vs* Aw-Sx	65	87	12	51
	Proteins reduced in abundance	Proteins increased in abundance	Proteins only detected in Aw-Sx	Proteins only detected in Aw-Se
Aw-Se *vs* Aw-Sx	27	49	18	4

Table 4
Changes in the relative abundance of proteins involved in different pathways of *Aspergillus westerdijkiae* MUM 16.142 growing on dry-cured ham-based medium at 20 °C for 14 days caused by *Staphylococcus xylosus* FHSCC Sx8.

Pathways	Identification	Protein	Log_2 fold change
Cell wall integrity	B8NWC7_ASPFN	Cofilin	Only in C[a]
	B8MYF4_ASPFN	Tubulin alpha chain	Only in C
	B8N080_ASPFN	Cell surface spherulin 4 family protein	Only in C
	B8NSH8_ASPFN	BAR adaptor protein RVS161, putative	Only in Sx[b]
	B8N2M1_ASPFN	Glutamine–fructose-6-phosphate transaminase	−0.456
	B8NRA6_ASPFN	Septin	−0.319
	B8NP29_ASPFN	Glycosidase	0.291
Biosynthesis of secondary metabolites	B8N6C9_ASPFN	Adenylyl cyclase-associated protein 1	Only in C
	B8N2C6_ASPFN	4-hydroxyphenylpyruvate dioxygenase	Only in C
	B8NBR1_ASPFN	Acetyl-CoA carboxylase, putative	Only in C
	B8N2C4_ASPFN	Homogentisate 1,2-dioxygenase	0.276
Stress response	B8N9C3_ASPFN	Superoxide dismutase	−0.167
	B8N103_ASPFN	Glutathione-S-transferase omega, putative	0.184
Carbohydrate metabolism	B8NJ30_ASPFN	Adenine phosphoribosyltransferase	Only in C
	B8MZY6_ASPFN	Glycosyl hydrolase, putative	Only in C
	XYNF3_ASPFN	Probable endo-1,4-beta-xylanase F3	−0.945
	B8N2M1_ASPFN	Glutamine–fructose-6-phosphate transaminase	−0.456
	B8NPD5_ASPFN	Phosphotransferase	−0.268
	B8MZQ0_ASPFN	Phosphoglycerate mutase (2,3-diphosphoglycerate-independent)	−0.224
	B8NBA2_ASPFN	l-fuconate dehydratase	0.211
	B8NGW0_ASPFN	Alpha-xylosidase, putative	0.218
	B8N2F2_ASPFN	Phosphoenolpyruvate carboxykinase	0.241

[a] Only in C: Protein only detected in the non-treated in *A. westerdijkiae*.
[b] Only in Sx: Protein only detected in *A. westerdijkiae* co-inoculated with *S. xylosus*.

Table 5
Changes in the relative abundance of proteins involved in different pathways of *Aspergillus westerdijkiae* MUM 16.142 growing on dry-cured ham-based medium at 20 °C for 14 days caused by *Staphylococcus xylosus* FHSCC Sx8 in comparison with the batch inoculated with *Staphylococcus equorum* FHSCC Se31.

Pathways	Identification	Protein	Log_2 fold change
Cell wall integrity	B8NNY0_ASPFN	Alcohol dehydrogenase, putative	−0.412
Biosynthesis of secondary metabolites	B8NWT2_ASPFN	Zinc-binding oxidoreductase, putative	−0.337
	B8N2C4_ASPFN	Homogentisate 1,2-dioxygenase	0.166
Stress response	B8MZG9_ASPFN	Hsp70 family chaperone Lhs1/Orp150, putative	0.244
Carbohydrate metabolism	B8NTE9_ASPFN	Glycosyl hydrolases family 32 superfamily	−0.796
	B8NWD3_ASPFN	Alpha-1,2-mannosidase family protein	−0.638
	BGLA_ASPFN	Probable beta-glucosidase A	−0.586
	B8MY83_ASPFN	Glycosyl hydrolase, putative	−0.411
	B8NZ24_ASPFN	GGY-family carbohydrate kinase, putative	−0.273
	B8NP68_ASPFN	6-phosphogluconate dehydrogenase, decarboxylating	0.104
	B8N9D5_ASPFN	Glucose-6-phosphate 1-epimerase	0.244

hand, proteins such as, zinc-binding oxidoreductase and homogentisate 1,2-dioxygenase related to the biosynthesis of secondary metabolites were altered in abundance in the presence of *S. xylosus* (Table 5).

In addition, an alteration in the abundance of seven proteins involved in carbohydrate metabolism was also observed in the presence of *S. xylosus*. Five of them were found in lower abundance and two proteins in higher quantity (Table 5).

3.5. Effect of S. xylosus against A. westerdijkiae in dry-cured ham

3.5.1. Effect on fungal growth

S. xylosus and *A. westerdijkiae* reached levels around 8 and 8.5 log cfu/g respectively, in both studied batches. Inoculation of *S. xylosus* with *A. westerdijkiae* did not cause a decrease in fungal growth in dry-cured ham after 14 days at 20 °C.

3.5.2. Effect on OTA production

The amount of OTA produced by *A. westerdijkiae* reached 161527 ng/g while in combination with *S. xylosus* the production was 53091 ng/g, showing a decrease of 67.19% (Fig. 5).

4. Discussion

The development of ochratoxigenic moulds on the surface of dry-cured ham poses a health risk to consumers. Previous works have studied the antifungal activity of autochthonous GCC+ such as *S. xylosus* and *S. equorum* (Cebrián et al., 2020, 2022) that could be useful as BCA throughout processing as they are present during all ripening stages. However, their modes of action have not been studied and further research is needed to optimise their application in dry-cured ham. For this reason, the present work assesses the potential modes of action for *S. xylosus* FHSCC Sx8 and *S. equorum* FHSCC Se31 against *A. westerdijkiae* MUM 16.142 on growth and OTA production.

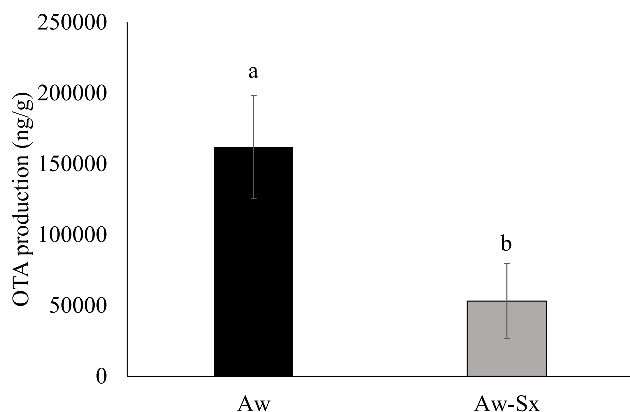

Fig. 5. Effect of *Staphylococcus xylosus* FHSCC Sx8 (Aw-Sx) on OTA production by *Aspergillus westerdijkiae* MUM 16.142 (Aw, untreated control) after 14 days at 20 °C in dry-cured ham. * Values of OTA concentration with different letters are significantly different ($p \leq 0.05$).

In this respect, the inhibition growth and OTA production of *A. westerdijkiae* by live cells of *S. xylosus* and *S. equorum* are consistent with those obtained by Cebrián et al. (2020), since *S. xylosus* Sx8 fully inhibited the growth of different ochratoxigenic, aflatoxigenic, and CPA-producing moulds on dry-cured ham-based medium. However, the inoculation of dead cells or extracellular compounds did not produce any effect in the *A. westerdijkiae* growth nor on the OTA production, as also occurred against *P. nordicum* (Cebrián, Roncero, Delgado, Rodríguez, & Núñez, 2023). Likewise, the inoculation of dead cells and cell-free broth from *Candida zeylanoides* did not lead to a reduction in the ochratoxigenic *P. nordicum* growth (Meftah et al., 2020). Therefore, it is confirmed that the inhibitory effect of both cocci observed in the present study is due to the activity of viable cells. However, this impact would be likely limited under real conditions, because although this high level of GCC+ can be reached in some ripening stages of dry-cured ham, at the end of the drying phase there is a load decrease (Rodríguez et al., 1994), and they would also compete with the wild microbiota.

The number of CSs metabolised by staphylococci compared to those metabolised by *A. westerdijkiae* gives information about their degree of dominance and part of their efficacy for use as BCA. It was found that both at 15 and 20 °C, *A. westerdijkiae* used a higher number of CSs than the two autochthonous staphylococci tested. The ochratoxigenic mould nutritionally dominated *S. xylosus* at both 15 and 20 °C as it has been observed for other toxigenic moulds against BCAs (Álvarez, Núñez, et al., 2021). However, although *A. westerdijkiae* also dominated *S. equorum* at 20 °C, the mould coexisted with this GCC+ at 15 °C. In this regard, a_w and temperature can influence the NOI of toxigenic and atoxigenic strains of *A. flavus* (Mohale, Magan, & Medina, 2013). These findings point to a lack of efficiency of *S. xylosus* at 15 or 20 °C and *S. equorum* at 20 °C as antagonist microorganisms against *A. westerdijkiae* when the nutritional competition is sought.

Regarding the volatile compounds generated by both *S. xylosus* and *S. equorum,* they significantly reduced the *A. westerdijkiae* growth at 15 °C, but not at 20 °C. In this sense, a previous work performed by Gong et al. (2020), demonstrated that *Staphylococcus saprophyticus* L-38 inhibits the growth of *A. flavus* by the production of 3,3-dimethyl.1,2-epoxybutane. Similarly, in another study carried out by Alijani et al. (2019), the volatile compounds produced by *Staphylococcus sciuri* MarR44 caused the inhibition of the mycelial growth and conidial germination of *Colletotrichum nymphaeae*. However, despite the slight reduction caused in the growth of *A. westerdijkiae*, the volatile compounds generated by the cocci did not affect the OTA production. In this regard, in a study performed by Cebrián, Roncero, Delgado, Núñez, and Rodríguez (2023) using the same GCC+, it was also found that the volatiles compounds produced by *S. xylosus* and *S. equorum* did not lead to OTA reduction, although there was a growth decrease at both assayed

temperatures. These results are not surprising since the relationship between mould growth and OTA production can be not linear (Sánchez-Montero, Córdoba, Peromingo, Álvarez, & Núñez, 2019) and even, the stress caused by certain BCAs can even lead to increase the mycotoxins production (Ponts, 2015).

Therefore, the mechanisms related to the dead cells, extracellular and volatile compounds, and competition for nutrients can be ruled out as the cause of the OTA inhibition by these staphylococci.

On the other hand, the interaction between *A. westerdijkiae* and *S. xylosus* or *S. equorum* was evaluated by a numerical scoring number (Magan & Lacey, 1984). Both cocci were able to deter the growth of *A. westerdijkiae* by contact at both 15 and 20 °C, suggesting that their mode of action could be attributed to competition by space, as it has been reported for *Penicillium chrysogenum* against *P. nordicum* (Álvarez, Núñez, et al., 2021). Overall, *S. xylosus* reduced OTA production even though it is not a good nutritional competitor, and no activity of its cellular components or production of extracellular compounds affecting OTA production by *A. westerdijkiae* has been detected. Therefore, its activity should be related to its capacity to affect the physiology of *A. westerdijkiae*. This led to perform proteomic analyses to unveil the changes occurred in *A. westerdijkiae* in the presence of *S. xylosus* with the ability to reduce OTA production and *S. equorum* without it.

When comparing the *A. westerdijkiae* co-inoculated with *S. xylosus* with the control batch, the mould proteome revealed that the CWI pathway was altered. In this sense, cofilin, tubulin alpha chain, and cell surface spherulin 4 family protein were only found in the control batch. In addition, glutamine-fructose-6-phosphate transaminase, and septin abundance were reduced by the effect of the coccus. All these proteins are involved in the normal modulation of actin binding, chitin formation or polysaccharides needed in the cell wall for the normal fungal growth (Bamford et al., 2015; Binder, Oberparleiter, Meyer, & Marx, 2010; Niggli, 2014; Oakley, 2004; Ram et al., 2004). These findings are likely the cause of the growth reduction observed in the dual assay.

Despite not finding altered PKS ERS domain proteins, as it has been described for other BCAs (Cebrián et al., 2023; Álvarez, Delgado, et al., 2021; Álvarez, Delgado, et al., 2022; Álvarez, Núñez, et al., 2022), the results showed alterations in proteins related to secondary metabolism. In this sense, three proteins were only detected in the control batch where the OTA production was higher. First, adenylyl cyclase associated protein that plays an important role in secondary metabolism in *Fusarium* species (Yang et al., 2022). The second protein is acetyl-CoA carboxylase that catalyses the reaction of addition of a bicarbonate group to acetate to obtain malonate, an OTA precursor (Ferrara, Gallo, Perrone, Magistà, & Baker, 2020; Wang et al., 2018). The third one is 4-hydroxyphenylpyruvate dioxygenase that catalyses the conversion of 4-hydroxyphenylpyruvate to homogentisate (Moran, 2005), involved in the L-phenylalanine pathway (Loughran, 2018; Palmer, Palmer, Jorth, & Whiteley, 2010). In this same way, the action of *S. xylosus* decreased the abundance of homogentisate 1,2-dioxygenase, an enzyme involved in the phenylalanine catabolic pathway (Loughran, 2018), similarly to what happened in *P. nordicum* co-cultured with this staphylococcus strain (Cebrián et al., 2023). Since the L-phenylalanine, an OTA precursor (Wang et al., 2016), is catabolised in other metabolic pathways, it would reduce its availability for OTA biosynthesis, which would impact on the lower production of this toxin.

On the other hand, proteins related to stress response were also altered. Superoxide dismutase was decreased in abundance by *S. xylosus*. This protein removes toxic radicals to biological systems, normally produced in cells. Glutathione-S-transferase omega was increased in abundance, as previously reported by Wang et al. (2021), where the proteomic profile of *Aspergillus ochraceus* in the presence of high levels of salt, which led to a reduction on OTA production (Wang et al., 2021).

Finally for this comparative, *A. westerdijkiae* proteome affected by *S. xylosus* presence revealed changes in the abundance of proteins linked to CS utilisation. The relationship between the results obtained in the NOI with the altered proteins linked to carbohydrate metabolism are apparently not consistent since *A. westerdijkiae* dominates nutritionally, but the proteins involved have decreased in abundance when the mould is confronted with *S. xylosus*. This apparent contradiction is likely due to the NOI test evaluates the metabolism of CSs independently for each microorganism as isolated entities, while in the dual test both microorganisms interact between them, being somewhat closer to the reality of the industry. Thus, it is likely that there are interactions between microorganisms that explain the decrease in proteins involved in carbohydrate metabolism despite the theoretical nutritional dominance of the mould.

When comparing the *A. westerdijkiae* co-inoculated with *S. equorum* batch, without significant reduction on OTA production, *versus* this mould co-inoculated with *S. xylosus* batch, with significant reduction on OTA production (Aw-Se *vs* Aw-Sx), differences in proteomes were noticed. These differences were not supported by the proteins directly linked to OTA production, but rather by proteins with apparently weaker impact on OTA production.

The proteome of the ochratoxigenic mould revealed that the CWI pathway was altered by *S. xylosus.* In this sense, alcohol dehydrogenase, putative was found decreased in abundance in the Aw-Sx batch, linked to OTA decrease. This is a key redox enzyme affecting the antioxidant capacity, cellular integrity and pathogenicity of fungi (Wang et al., 2023).

Furthermore, two proteins related to secondary metabolism were also affected. In this regard, a drop in the abundance of zinc-binding oxidoreductase, putative and an increase of homogentisate, 1,2- dioxygenase were observed in the Aw-Sx batch. Although no studies have been found that support a direct relationship with OTA, the protein zinc-binding oxidoreductase is related to the production of other toxins, such as gliotoxins by *Aspergillus fumigatus*, where a lower gliotoxin production was obtained with a higher amount of zinc (Traynor et al., 2021). On the other hand, the increase in the abundance of homogentisate 1,2-dioxygenase, similarly to the observed in the comparison between Aw *vs* Aw-Sx, can be linked to the L-phenylalanine metabolism (Loughran, 2018; Palmer et al., 2010; Wang et al., 2016). Then, as the abundance of homogentisate 1,2-dioxygenase is elevated, as discussed for the Aw *vs* Aw-Sx comparison, would entail a higher L-phenylalanine catabolism, and therefore it would not be sufficiently available to give rise to OTA, which contributes to explain the reduction of this toxin by *S. xylosus*.

On the other hand, a protein related to the stress response was found: Hsp70 family chaperone Lhs1/Orp150, putative was increased in abundance in the Aw-Sx batch. This fact is related to a previous study by Delgado et al. (2015) in which this protein was also found to be increased in the presence of an antifungal protein, which, by reducing the growth of *A. flavus*, provoked stress in the mould.

Finally, the proteome of *A. westerdijkiae* in the presence of *S. equorum versus* this mould affected by *S. xylosus* (Aw-Se *vs* Aw-Sx) revealed changes in the abundance of proteins related to carbon source utilisation, despite being in the same niche (being both cocci nutritionally dominated by *A. westerdijkiae*). Nevertheless, the degree of relative changes in this group of proteins is moderate, since no qualitative differences (usually linked to high changes in amount) were found (Table 5).

Furthermore, taking into account that the study of mode of action of these microorganisms is carried out in a culture medium and therefore, under more controlled conditions, it was crucial to evaluate them in the product itself, as a more complex matrix can lead to differences in the results. Therefore, the antifungal and antiochratoxigenic effect of *S. xylosus* was investigated in dry-cured ham pieces, in which OTA produced by *A. westerdijkiae* was reduced in the presence *S. xylosus* at 20 °C, as in the dual culture assay in DHA, although there was no reduction in fungal growth. However, the reduction of OTA was not fully efficient, similarly to the observed in the assays performed on DHA. In this regard, it is possible that co-action with another BCA may result in further inhibition of this mycotoxin production, as also occurred for *P. nordicum* when treated with *D. hansenii* and *Penicillium chrysogenum*

(Cebrián et al., 2019).

5. Conclusions

The most conducive temperature to reduce the OTA production by GCC+ seems to be at 20 °C, although *S. equorum* does not work. The best results obtained with *S. xylosus* are based on competition for space, causing alterations in the mould proteome with changes in the abundance of proteins related to CWI pathway, OTA synthesis and carbohydrate metabolism. In order to enhance its anti-ochratoxigenic effect, the incorporation of *S. xylosus* would be more favourable in the drying-maturing stage in the industry, where the temperature of 20 °C is reached and maintained. Thus, *S. xylosus* could be useful within a HACCP framework to minimise the hazard of OTA production by *A. westerdijkiae* in dry-cured ham. Finally, this study paves the way to propose the use of this coccus together with another BCA, preferably with impact on different pathways of *A. westerdijkiae* to those revealed for *S. xylosus* mode of action, to maximize its effect.

This study has been carried out under controlled conditions, making arduous the translation of the findings to a complex meat product, populated by wild microbiota and longer ripening with varying environmental factors. Therefore, similar studies in dry-cured ham processed in industrial facilities are required in the future to corroborate these results. Nevertheless, these findings take a step forward for a better and more comprehensive understanding of the impact of cocci biocontrol agents with dissimilar efficacy on *A. westerdijkiae* inhibition.

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CRediT authorship contribution statement

Eva Cebrián: Formal analysis, Investigation, Writing – original draft. **Mar Rodríguez:** Conceptualization, Funding acquisition, Validation, Writing – review & editing. **Micaela Álvarez:** Investigation. **Josué Delgado:** Conceptualization, Data curation, Writing – review & editing. **Félix Núñez:** Conceptualization, Funding acquisition, Validation, Writing – review & editing.

Declaration of competing interest

The authors declare that they have no known competing financial 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.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.lwt.2023.115576.

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