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Inhibition of *A. carbonarius* growth and reduction of ochratoxin A by bacteria and yeast composites of technological importance in culture media and beverages

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ABSTRACT

Five composites of yeast and six of bacterial isolates from fermented products were studied, in order to assess their ability to inhibit *Aspergillus carbonarius* growth and reduce OTA concentration in culture media and beverages. The antagonistic effect of the above composites against *A. carbonarius* growth was studied in synthetic grape medium of pH 3.5 and a_w 0.98, 0.95, 0.92 after incubation at 25 °C. Different combinations of initial inocula of bacteria or yeast composites and fungi were used (10^2 cfu/mL vs 10^5 spores/mL; 10^5 cfu/mL vs 10^2 spores/mL; and 10^5 cfu/mL vs 10^5 spores/mL). Regarding the OTA reduction experiment, 10^3 and 10^7 cfu/mL of the bacteria and yeast composites were inoculated in liquid media of different pH (3.0, 4.0, 5.0, and 6.1 or 6.5) and initial OTA concentration (50 and 100 μg/L) and incubated at 30 °C. Moreover, grape juice, red wine, and beer were supplemented with 100 μg/L of OTA and inoculated with composites of 16 yeasts (16YM) and 29 bacterial (29BM) strains (10^7 cfu/mL) to estimate the kinetics of OTA reduction at 25 °C for 5 days. Fungal inhibition and OTA reduction were calculated in comparison to control samples. None of the bacterial composites inhibited *A. carbonarius* growth. The high inoculum of yeast composites (10^5 cfu/mL) showed more efficient fungal inhibition compared to cell density of 10^2 cfu/mL. All yeast composites showed higher OTA reduction (up to 65%) compared to bacteria (2–25%), at all studied assays. The maximum OTA reduction was obtained at pH 3.0 by almost all yeast composites. For all studied beverages the decrease in OTA concentration was higher by yeasts (16YM) compared to bacteria (29BM). The highest OTA reduction was observed in grape juice (*ca* 32%) followed by wine (*ca* 22%), and beer (*ca* 12%). The present findings may assist in the control of *A. carbonarius* growth and OTA production in fermented foodstuffs by the use of proper strains of technological importance.

1. Introduction

Some filamentous fungi of the genera *Aspergillus* and *Penicillium* are of high impact for food safety due to their ability to produce a toxic secondary metabolite, ochratoxin A (OTA). OTA is frequently detected in foods of wide consumption, such as cereals (58% of total OTA intake), wine (21%), grape juice (7%), and coffee (5%) (JECFA, 2001). The effects of OTA may be teratogenic, immunosuppressive and carcinogenic, while the last two decades, OTA has been classified as a potential human carcinogen (Class 2B) (IARC, 1993; Murphy et al., 2006).

In order to control OTA in foods, pre-harvest methods should be applied first to prevent or eliminate fungal contamination in the field and if the contamination persists after this phase, then fungal growth and OTA produced levels should be controlled through post-harvest interventions. In the post-harvest phase, storage and processing are the major stages where OTA can be prevented. If the applied procedures prove to be insufficient, detoxification of OTA is a possible alternative method. Specifically, OTA detoxification methods are classified into physical, chemical or (micro) biological, aiming to reduce or eliminate OTA by destroying, modifying or absorbing it (FAO/WHO/UNEP, 1999). The last decade researchers have shown increasing interest, for the biological methods to control OTA in foods, by studying bacteria, yeasts and non-toxic fungi for their potential ability to inhibit growth of ochratoxigenic fungi, as well as to decrease OTA, through degradation or binding, to acceptable by the legislation levels (2–10 μg/L) (Bleve et al., 2006; del Prete et al., 2007; EC No, 1881/2006; Moruno et al., 2005; Varga et al., 2000).

Yeasts and bacteria (mainly lactic acid bacteria-LAB) constitute promising potential biocontrol agents of fungal growth or OTA occurrence due to their ability to grow and survive in food products under a wide range of environmental conditions, either as a part of the indigenous microflora or supplemented as starter or protective cultures (Dalié et al., 2010; Wilson and Wisniewski, 1989). Numerous strains of bacteria and yeasts have been studied, individually, and shown positive effect on inhibition of ochratoxigenic fungi or on OTA reduction (Cecchini et al., 2006; Fuchs et al., 2008; Masoud and Høj Kaltoft,

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2006; Piotrowska and Zakowska, 2005). However, considering that indigenous or exogenously added cultures often contain more than one species or strains, there is a need for studying the behavior of microorganisms in mixed cultures. Such information is limited in literature but necessary in order to clarify whether the use of certain composites of strains or species may increase the possibility for inhibition of ochratoxigenic fungi or for OTA reduction. In addition, the effect of critical factors e.g. pH, a_w, initial toxin concentration, and initial cell density on fungal inhibition and OTA detoxification dynamics, in culture media or in real food products, have not been sufficiently studied (Bejaoui et al., 2004; Cecchini et al., 2006; Fuchs et al., 2008). The knowledge of the effect of the aforementioned factors on microorganisms used for OTA control is required, since such information could possibly assist in prevention of fungal growth and OTA production in the complex ecosystem of fermented e.g., wine, beer, bread etc. or non fermented foods e.g. juices.

Considering the above, the objectives of this study were: i) to assess the competitive activity of yeast and bacterial composites of technological importance, used as protective cultures, on growth of *Aspergillus carbonarius* in response to a_w and different combinations of initial ratios of cells over spores densities, ii) to evaluate the ability of the aforementioned microorganisms to reduce different initial OTA concentrations in liquid culture media of different pH and low or high initial cell density, and iii) to study their ability to reduce the toxin, in beverages such as grape juice, red wine, and beer as an extra prevention measure, before the final products were placed to the market.

2. Materials and methods

2.1. Microorganisms and inocula preparation

Twenty nine (29) bacterial strains were isolated from different products of animal and plant origin such as fermented sausage, sourdough, flour, aniseed, basil, while sixteen (16) yeast isolates were obtained from different wine batches (Tables 1 and 2). Yeast and bacterial strains were obtained from the microbial stock collections of Laboratories of Food Microbiology & Biotechnology and Food Quality Control & Hygiene of Agricultural University of Athens. All strains were coded and stored in glycerol at −22 °C.

Overnight cultures of individual yeast (25 °C, 24 h) and bacteria (30 °C, 24 h) strains were harvested by centrifugation (3000 *g*, 4 °C, 15 min). The supernatants were discarded and cells in the pellets were resuspended in Maximum Recovery Diluent (MRD). The washing step was repeated twice. Preliminary experiments showed that OTA reduction was in the range of 5 to 15% when bacteria and yeasts were inoculated individually (data not shown). Moreover, considering that in most of the cases, cultures, either indigenous or exogenously added (i.e., protective cultures), consist of more than one species, the selected bacteria and yeasts were studied in composites of 2–8 isolates or in a composite of all isolates per microorganism category (i.e., 16YM and 29BM) (Maragkoudakis et al., 2009; Vermeiren et al., 2004). Specifically, bacteria and yeast isolates were divided in five (BM, PdM, SM, WM, LbM) and four (YM1, YM2, YM3, YM4) composites respectively (Tables 1 and 2). Bacteria were grouped according to their genera. Composition of yeast mixtures and the number of species in each mixture have been decided based on preliminary experiments (data not shown). In order to decide which mixtures will be first studied, the observations of Nisiotou et al. (2007) were taken into account and yeasts isolated from different wine variety such as Sefka, Moschofilero, Mavroliatis, and Limnio during fermentation (beginning, middle, end) were used (Drosinos et al., 2007; Nisiotou et al., 2007; Paramithiotis et al., 2010).

Spore suspension of *A. carbonarius* (ATHUM 5659) was prepared by collecting spores from 7-day old colonies (grown on Malt Extract Agar; MEA at 25 °C) in distilled sterilized water supplemented with 0.01% Tween 80 to assist in the dispersal of conidia (10^7 spores/mL). Fungal cultures were stored on MEA plates (Lab M Limited, United Kingdom) at 2–8 °C and subcultured once a week.

Table 1
Overview of bacteria isolates used in the present study.

Bacterial species	Code	Origin (reference)
Bacillus thuringiensis (7)	BM	Aniseed, basil and dough (unpublished data)
Pediococcus pentosaceus (6)	PdM	Flour, sourdough, and dough (Paramithiotis et al., 2010)
Streptococcus salivarius (6)	SM	Sourdough and dough (unpublished data)
Weissella cibaria (8)	WM	Flour, sourdough, and dough (unpublished data)
Lactobacillus sakei (1)	LbM	Fermented sausages (Drosinos et al., 2007)
Lactobacillus casei (1)		Fermented sausages (Drosinos et al., 2007)
All bacteria strains	29BM	

Numbers in parentheses indicate the number of strain tested.

Table 2
Overview of yeast isolates used in the present study.

Yeast species	Code	Origin (Nisiotou et al., 2007)	Fermentation stage
Hanseniaspora guilliermondii	YM1	Limnio	BF; MF
Kluyveromyces dobzhankii		Moschofilero	MF
Pichia fermentas		Mavroliatis	BF; MF
Issatchekia occidentalis		Sefka	BF; MF
Metschnikowia pulcherrima	YM2	Mavroliatis	BF
Hanseniaspora uvarum		Sefka	BF; MF
Issatchenkia terricola		Moschofilero	BF; MF
Zygosaccharomyces bailii		Mavroliatis	BF; MF; EF
Zygosaccharomyces bailii	YM3	Moschofilero	BF; MF; EF
Kazachstania hellenica (2 strains)		Mavroliatis;Sefka	MF; EF
Hanseniaspora opuntiae		Limnio	BF; MF
Saccharomyces cerevisiae	YM4	Sefka; Moschofilero	MF; EF
Pichia guilliermondii		Mavroliatis	BF; MF
Lachencea thermotolerans		Limnio	BF
Issatchenkia orientalis		Mavroliatis	BF; MF; EF
All yeast strains	16YM		

BF: beginning of fermentation; MF: middle of fermentation; EF: end of fermentation.

2.2. A. carbonarius inhibition experiment

The experiment was performed on Synthetic Grape Medium (SGM), a culture medium that sufficiently simulates authentic grape juice and ensures the quantification of *A. carbonarius* inhibition by bacteria and yeast mixtures under strictly controlled conditions (i.e., acidity, ionic strength, carbohydrate concentration, etc.). An extra advantage of using a solid substrate such as SGM was the easier observation of *A. carbonarius* inhibition zone caused by the immobilized yeast and bacteria colonies resulting in more accurate quantification (diameter) of fungal inhibition compared to a more turbid natural substrate such as grape juice. This has also been exemplified in previous reports (Belli et al., 2005; Tassou et al., 2009). Two types of SGM with 0.7 and 2.5% agar were produced. Both media were prepared by adding D(+)-glucose, 70 g; D(−)-fructose, 30 g; L(−)-tartaric acid, 7 g; L(−)-malic acid, 10 g; $(NH_4)_2SO_4$, 0.67 g; $(NH_4)_2HPO_4$, 0.67 g; KH_2PO_4, 1.5 g; $MgSO_4 \cdot H_2O$, 0.75 g; NaCl, 0.15 g; $CaCl_2$, 0.15 g; $CuCl_2$, 0.0015 g; $FeSO_4 \cdot 7H_2O$, 0.021 g; $ZnSO_4$, 0.0075 g; (+) catechin hydrate, 0.05 g, to 1000 mL of water. The pH of SGM was increased to 3.5 by adding 2 N KOH. Addition of different amounts of glycerol was also performed to adjust a_w of the media to 0.98, 0.95 and 0.92. Glycerol was used to adjust a_w, rather than other agents such as sugars, or NaCl due to its neutral behavior to fungal metabolism. Both, pH and a_w values of culture media were measured after

autoclaving using a digital pH meter (pH 526, Metrohm Ltd, Switzerland) and a water activity meter (Hydrolab rotronic, Basserdorf, Switzerland) respectively.

Six (6) mL of SGM with 0.7% agar and 1 mL of each yeast or bacterial composite suspension were mixed and poured on 15 mL of the SGM (2.5% agar) solidified agar medium (Bleve et al., 2006). Any differences in a_w caused by the addition of yeast or bacterial composite suspensions have been recorded in preliminary experiments and taken into account in order to be balanced by adding the appropriate amounts of glycerol. Following solidification, aliquots of *A. carbonarius* spore suspension were centrally spotted on the surface of agar. The three different combinations of initial inocula of bacterial or yeast composites and *A. carbonarius* were, 10^2 cfu/mL–10^5 spores/mL, 10^5 cfu/mL–10^2 spores/mL, and 10^5 cfu/mL–10^5 spores/mL. The volume of *A. carbonarius* inoculum size was low to cause no changes in a_w. Specifically, aliquots of 22 μL from 10^5 spores/mL MRD and from 10^8 spores/mL MRD (concentrated inoculum) were used to succeed the tested concentrations of 10^2 and 10^5 spores/mL SGM respectively. This parameter was studied, in order to assess the ability of these composites to inhibit *A. carbonarius* under different conditions of competition. Plates not inoculated with bacteria or yeasts were used as controls. All samples were incubated for 5 days at 25 °C, which is the optimum temperature for *A. carbonarius* (ATHUM 5659) growth (Kapetanakou et al., 2010; Tassou et al., 2009). The experiment was performed twice and at least duplicate samples were analyzed in each replicate. Fungal growth inhibition was determined after the end of incubation, according to the equation:

$$\%\ \text{Inhibition} = 1-(\text{diameter of fungal colony growth on medium with microorganisms/diameter of fungal colony growth on medium without microorganisms})\times 100. \quad (1)$$

2.3. OTA reduction experiment

2.3.1. Culture media

The potential of yeast and bacterial composites for OTA reduction was evaluated in yeast medium (YM; initial pH 6.5; a_w 0.99) and MRS broth (initial pH 6.1; a_w 0.99), respectively. Yeast medium was prepared by dissolving 0.3 g Malt Extract (Merck, Darmstadt, Germany), 0.3 g Yeast Extract (Lab M Limited, United Kingdom), 0.5 g Peptone Universal (Merck, Darmstadt, Germany), 1 g Glucose (Riedel de Haan AG, Germany) in 100 mL distilled water. The pH of both media was adjusted to 3.0, 4.0, and 5.0 by adding 6 N HCl. The pH was measured after autoclaving (121 °C; 15 min). Due to the fact that different fungi species are known to produce OTA in different foodstuffs, i.e. *A. carbonarius* on wine and grape juice and *Penicillium verrucosum* or *Aspergillus ochraceus* on grains and subsequently on beer, OTA was added to the media instead of inoculating an ochratoxigenic fungus. Specifically, after sterilization, the appropriate volume of culture media was supplemented with aliquots of OTA (10.20 μg/mL, Biopure Corporation, USA), to obtain two different concentrations of 50 and 100 μg/L. Five (5) mL from both laboratory media was aseptically poured in 15 mL plastic screw-cap sterile tubes.

Each species of 29 bacteria and 16 yeasts was activated individually, centrifuged, washed twice and resuspended in MRD (Section 2.1). Following resuspension, composites were produced accordingly to Tables 1 and 2 by using equal volumes of each species or strain. Decimal dilutions were carried out to reach a low (10^3 cfu/mL) and a high (10^7 cfu/mL) inoculum, studying whether their ability for OTA reduction is affected by the initial cell density. The inoculation of culture media supplemented with OTA was not carried out by adding liquid inocula, so as not to dilute the final OTA concentration. Following dilutions, yeast or bacterial composite suspensions were centrifuged again. The cell pellets were finally resuspended in culture media of different pH values supplemented with OTA (50 and 100 ppb). The incubation was performed at 30 °C, which is a temperature close to the optimum for both microorganism categories. The duration of incubation was decided by preliminary experiments (data not shown), which indicated that the maximum OTA reduction occurred on day 2 for yeasts and day 5 for bacterial composites. Positive controls, i.e., medium with 50 or 100 μg/L OTA, but without microorganism (yeasts or bacteria) were used to calculate the percentage of OTA reduction, while a negative control containing microorganism without OTA was also used to determine the initial cell density (cfu/mL).

2.3.2. Beverages

Volumes of 200 mL of Greek commercial beverages such as grape juice (pH 3.3 and a_w 0.96), red wine (pH 3.6 and a_w 0.96) and beer (pH 4.4 and a_w 0.97) were supplemented with *ca* 2 mL from a concentrated solution of OTA (10.20 μg/mL, Biopure Corporation, USA) to obtain a final OTA concentration of 100 μg/L (Table 3). The selection of the beverages was mainly based on the frequent detection of OTA and their wide consumption. Especially grape juice constitutes a special product due to its wide consumption by children. Therefore it is important to find ways to reduce the potential occurrence of OTA in this product. Some of the tested species e.g. *Pediococcus pentosaceus*, the two *Lactobacilli*, *Streptococcus* sp. and *Saccharomyces cerevisiae* have been successfully used as protective cultures of fermented (wine and beer) or non-fermented (e.g., grape juice) food (Legras et al., 2007; Maragkoudakis et al., 2009; Vermeiren et al., 2004). This type of bacteria or yeasts may possess anti-microbial activity against spoilage or pathogenic microorganisms without altering the sensory properties of foodstuff (Gaggia et al., 2011). All products were not pasteurized in order to also study the role of endogenous microflora. In each beverage, the composites of 16 yeasts (16YM) or 29 bacteria (29BM) at the level of 10^7 cfu/mL were added. Inocula preparation and addition into beverages carried out according to Section 2.3.1. A negative control, namely an OTA contaminated beverage without microorganisms was used to ensure that OTA reduction was primarily due to the inoculated microorganisms and not due to the endogenous microflora of beverages. Given that ochratoxigenic fungi mainly occur either in the field (pre-harvest) or during storage of harvested products (post-harvest), OTA primarily constitutes a problem in raw materials (Duarte et al., 2010; Hocking et al., 2007). However it is possible for OTA to be detected during wine or beer processing (fermentation or malting) and storage of the final product (beverages) (Mateo et al., 2007). Assuming that raw products, such as grape berries or barley, have been contaminated by ochratoxigenic fungi and that after fermentation (wine), processing (grape juice) or malting (beer), the detected OTA concentrations in the final products were over the legislation limits, yeast and bacteria were added to the final products as potential protective cultures, in order to evaluate whether they may reduce a potential pre-existing OTA during storage of the products. Controls and inoculated samples were incubated at 25 °C, considering that all three beverages are usually being stored at ambient conditions, before placing to the market. Furthermore, our present aim was to evaluate whether the composites proposed are effective in reducing OTA under conditions optimal for the cultures and then further studies may follow to optimize time and temperature of storage that might deliver the maximum OTA reduction.

Table 3
Composition and basic physicochemical characteristics of the beverages tested.

[a]Foodstuff	Ingredients	pH	a_w	Alcohol (% vol/vol)
Grape juice	Water, grape juice (20%), sugar, citric acid	3.3	0.96	–
Red wine	–	3.6	0.96	12
Beer	Water, barley malt, hops, yeast	4.4	0.97	5

[a] Without preservatives.

Samplings were performed on days 0, 2, and 5. All experiments were performed twice and at least duplicate samples were analyzed in each replicate. The percentage of OTA was calculated using the equation:

$$\% \text{ Reduction} = [1-(C_{OTA}\text{of sample}/C_{OTA}\text{of control})]\times 100. \quad (2)$$

2.4. Detection of OTA by HPLC–FLD

After the end of incubation, all samples (culture media and beverages) were centrifuged (3000 *g*, 4 °C, 20 min) and supernatants were collected for OTA determination. On both experiments, clean up of samples took place by immunoaffinity columns (OchraStar™, R-Biopharm AG, Darmstadt, Germany) strictly according to manufacturer's instructions. The samples were stored at −18 °C until HPLC analysis.

OTA detection and quantification were carried out accordingly to Möller and Nyberg, 2003. Specifically, chromatographic separation was performed under isocratic conditions at a flow rate of 1 mL/min of the mobile phase (water/acetonitrile/acetic acid: 49/49/2) using a fluorescence detector (LaChrom Hitachi, L-7485, Merck, Germany) (k_{exc} 333 nm; k_{em} 460 nm). A calibration curve was performed in the range of 0–120 μg/L OTA in culture media and beverages, to quantify the toxin. It must be also reported that 5 and 50 μg/L of OTA were prepared to determine the recovery percentage of OTA on laboratory media and beverages, while the detection limit of OTA was 1 ppb.

2.5. Measurement of pH, a_w and viable count

Measurements of pH and a_w of beverages were performed at every sampling. On day 0, 1 mL from each sample (control and inoculated) was used for enumeration of initial inoculation level and the level of the natural microflora of beverages, while the population of yeasts, bacteria and total viable counts were enumerated at all sampling days on YGC agar (25 °C, 3–5 days), MRS agar (30 °C, 5 days) and PCA (30 °C, 3 days) respectively, following serial dilutions in MRD.

2.6. Statistical analysis

OTA reduction was estimated by the Univariate analysis of the SPSS, Version 16.0 (SPSS Inc., Chicago, IL, USA). Descriptive statistics (means and standard deviations) were computed and analyses of variance were performed for the fixed effects and their interactions. Differences are reported at a significance level of 95%.

3. Results

3.1. Fungal inhibition

The low pH of SGM (pH 3.5) did not allow bacterial composites to grow and inhibit *A. carbonarius*, therefore this part of the study was focused on yeast composites. Factors such as the type of yeast composite, a_w, and combination of initial inocula of yeasts and fungi affected significantly OTA reduction ($p<0.05$). Fungal growth was suppressed at the following order of relative inoculation levels of yeasts and *A. carbonarius*: 10^5 cfu/mL-10^2 spores/mL (61–93%) > 10^5 cfu/mL-10^5 spores/mL (49–85%) > 10^2 cfu/mL-10^5 spores/mL (0–48%), regardless of the yeast composite (Fig. 1). The highest fungal inhibition was obtained at a_w 0.95, by almost all yeast composites and relative initial inocula of yeasts and the fungus. Visual observations indicated that at the lower inoculation levels of yeast composites (10^2 cfu/mL), individual yeast colonies occurred (Fig. 2a), whereas at higher inoculation levels (10^5 cfu/mL), uncountable colonies formed a continuous layer on agar surfaces, which resulted in a clear inhibitory zone of fungal growth (Fig. 2b and c).

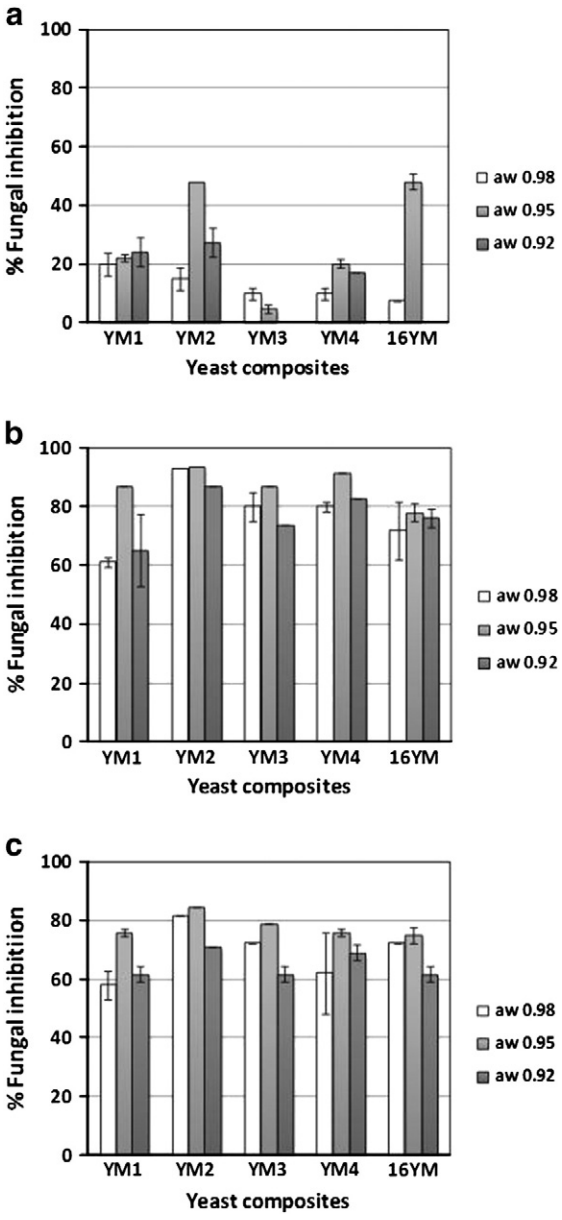

Fig. 1. Percentages of growth inhibition of *Aspergillus carbonarius* in SGM agar plates (pH 3.5; a_w 0.98, 0.95, and 0.92), inoculated by five yeast composites (YM1, YM2, YM3, YM4, 16YM), and incubated at 25 °C for 5 days. Different ratios of initial levels of yeasts and fungi were studied: (a) 10^2 cfu/mL vs 10^5 spores/mL; (b) 10^5 cfu/mL vs 10^2 spores/mL; and (c) 10^5 cfu/mL vs 10^5 spores/mL.

3.2. OTA reduction

3.2.1. Culture media

Preliminary experiments showed that the amounts of OTA absorbed by biomass were negligible (data not shown) compared to those recovered from the culture supernatants. Recovery percentages of OTA in MRS and YM broth were in the range of 92–107% and 88–113%, respectively. Bacterial strains showed low ability for OTA reduction, ranging from 2 to 25% at all examined combinations of pH and initial OTA concentration (Fig. 3a and c). Statistical analysis showed that the ability of all the bacterial composites for OTA reduction was slightly affected by the OTA initial concentration ($p=0.04$). On the contrary, factors such as pH, type of bacterial composite, and all their interactions significantly affected OTA reduction ($p<0.05$). Specifically, the increase in pH of the medium inoculated with the mixtures of *Bacillus thuringiensis* (BM), *P. pentosaceus* (PdM), and

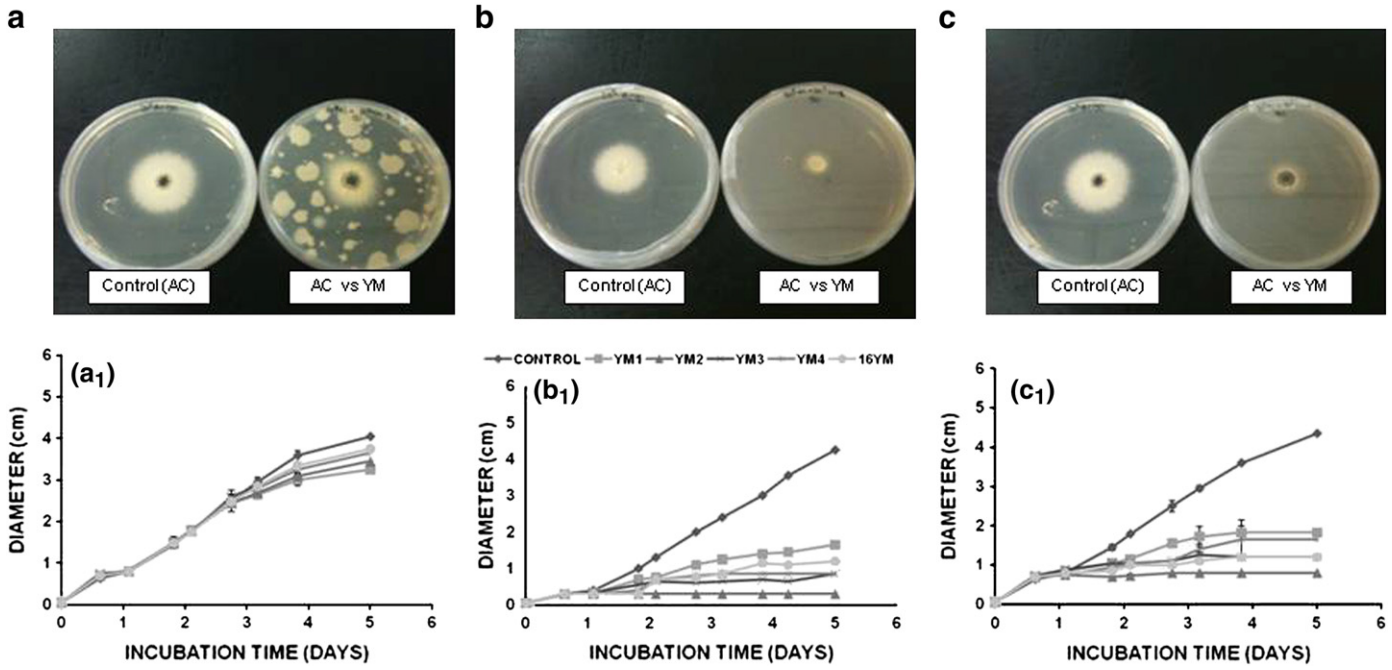

Fig. 2. Visual observation of inhibitory zone of *A. carbonarius* (AC) due to growth of yeast mixtures (YM) (right Petri dish) and graphical illustration of *A. carbonarius* diameter co-cultured with yeast mixtures, in SGM (pH 3.5, a_w 0.98) in comparison to control samples (inoculation only with *A. carbonarius*) (left Petri dish) during incubation at 25 °C for 5 days. The different ratios of initial inoculation levels of yeasts over fungal spores were: (a; a_1) 10^2 cfu/mL vs 10^5 spores/mL; (b; b_1) 10^5 cfu/mL vs 10^2 spores/mL; and (c; c_1) 10^5 cfu/mL vs 10^5 spores/mL.

Lactobacillus sp. (LbM), showed a slight but significant increase in OTA reduction (up to 25%), regardless of OTA initial concentration. At pH 3.0, no growth was observed by all the studied bacterial composites and thus no OTA reduction was reported (Fig. 3a and c).

Contrary to bacteria, yeast composites showed markedly higher detoxification activity, achieving as high reductions of OTA as 65% (Fig. 3b and d). The highest percentages of OTA reduction were obtained by the composites of YM1 (*ca* 53%), YM4 (*ca* 44–50%), and 16YM (*ca* 61–65%) at pH 3.0, regardless of OTA initial concentration. Moreover, OTA reduction was significantly affected by the yeast composite, the initial concentration of OTA in the medium, the pH and all their interactions ($p<0.05$). OTA reduction levels increased as pH of

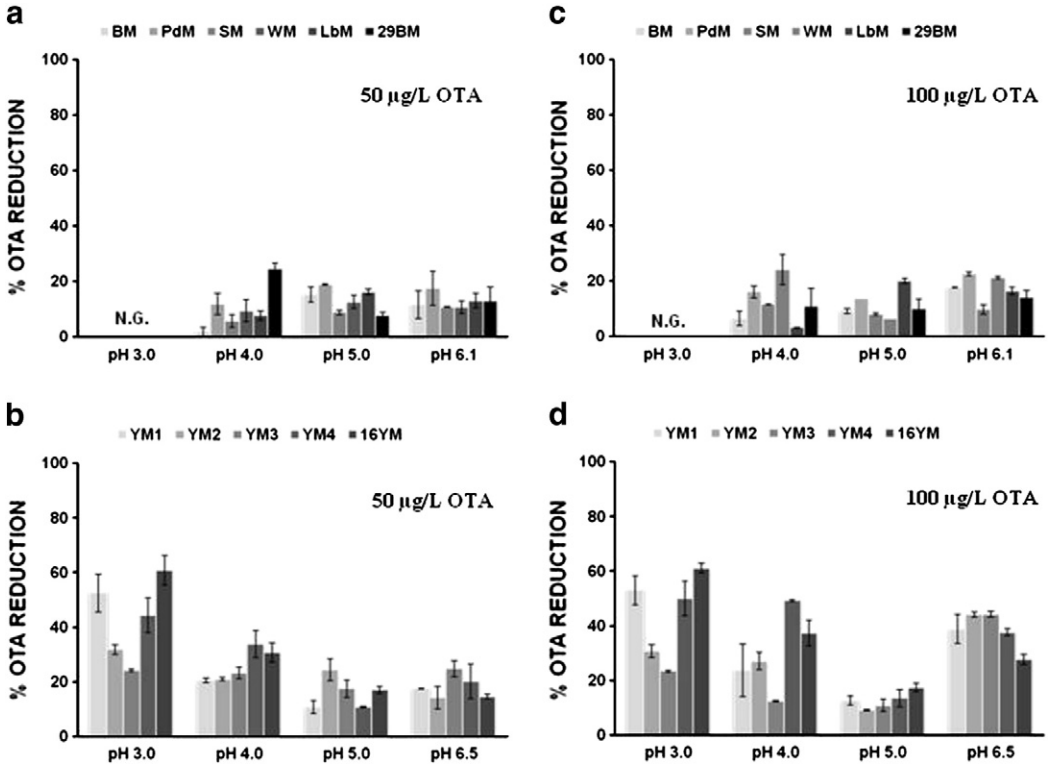

Fig. 3. Percentages of OTA reduction in liquid media of pH (3.0, 4.0, 5.0, 6.1 or 6.5) and OTA initial concentration (50 and 100 µg/L) inoculated with different composites (10^3 cfu/mL) of: (a, c) bacteria (BM, SM, PdM, WM, LbM, 29BM); and (b, d) yeasts (YM1, YM2, YM3, YM4, 16YM), after incubation at 30 °C for 5 and 2 days respectively.N.G.: no growth was observed.

the medium decreased, at almost all yeast composites (except for YM2 and YM3). Regarding the effect of OTA initial concentration on toxin reduction, the percentages between the two levels studied were similar, at almost all studied pH values, except for pH 6.5, where all studied composites showed higher percentages of reduction to 100 μg/L initial concentration compared to 50 μg/L.

In order to assess the effect of the initial inoculum size of yeast or bacterial composites in culture media on OTA reduction, we conducted experiments with two initial inoculation levels, namely 10^3 and 10^7 cfu/mL. Composites of 16 yeasts (16YM) and 29 bacteria (29BM) strains were studied. 16YM showed higher OTA reduction ability (14–68%) compared to 29BM (8–27%) (Fig. 4). Regarding the yeast composite, the percentages of OTA reduction increased by decreasing the pH of the medium, achieving the highest % reduction at pH 3.0. The latter result was observed on both studied (low and high) initial inocula. Initial cell density of 16YM seemed to have no specific effect on OTA reduction, and the observed differences may be in the range of standard deviation (Fig. 4b and d). On the contrary, cell density of 10^7 cfu/mL for the 29BM indicated, in some cases, significant higher (2 to 3-fold) ability for reduction, e.g. at pH 5.0, compared to low inoculum level (10^3 cfu/mL) (Fig. 4a and c).

3.2.2. Beverages

Among the three studied beverages, the maximum OTA reduction was observed in grape juice (up to 32%) followed by red wine (up to 22%) and beer (up to 12%) (Fig. 5). In agreement with the observations on culture media, the higher OTA reductions were achieved by the composite of 16 yeasts than by the 29 bacteria (2–8% OTA reduction) isolates, in all beverages. OTA reduction in control samples was negligible in wine and beer (1–4%), while in the case of grape juice, the corresponding percentages of OTA reduction were notable (1–21%). The latter order of magnitude was close to that obtained by 16YM (15–32%). Recovery percentages of OTA from grape juice, red wine, and beer were in the range of 107–114%, 95–119%, and 98–112% respectively. Moreover, a_w of all beverages showed a variation of 0.95–0.97 during incubation, while pH was varying in the range of 3.3–3.5, 3.5–3.6, and 4.4–4.5 for grape juice, red wine, and beer respectively. Regarding the initial population of the natural microflora, the total viable counts (TVC) on grape juice were *ca* 10^4 cfu/mL, while $<10^2$ cfu/mL on red wine and beer. During the incubation, the maximum enumerated TVC of control samples were 10^6–10^7 cfu/mL for grape juice and beer, while 10^4 cfu/mL for red wine, while the populations of the inoculated samples with yeast or bacteria composites remained stable around $7\log \pm 0.5$ during the 5-day storage period of all beverages.

4. Discussion

4.1. Fungal inhibition

Fungal biocontrol by using non-toxic competitive agents such as bacteria or yeast strains is a potentially useful and effective strategy to control OTA in foods. Several literature reports indicate that LAB, including some of the strains tested in our study may restrict fungal growth. Specifically, studies have reported that *Lactobacillus rhamnosus, L. plantarum* or *P. pentosaceus* and *Weissella cibaria* showed clear zones of inhibition for *Penicillium expansum* or *P. nordicum* respectively (Rouse et al., 2008; Schillinger and Villarreal, 2010). Moreover, *Bacillus licheniformis* has also been found capable of inhibiting growth of *Aspergillus westerdijkiae* (Petchkongkaew et al., 2008). However, the bacteria tested in the present study did not prove to be effective inhibitors for *A. carbonarius*, even though the majority of them belong to probiotics (Gaggia et al., 2011). A limited number of reports have shown that a good selection of bacteria or yeasts could allow the control of fungal growth and improve the shelf life of many fermented products reducing by this way health risks due to exposure to mycotoxins, therefore microorganisms having protective properties were decided to be tested in the present study (Gourama and Bullerman,

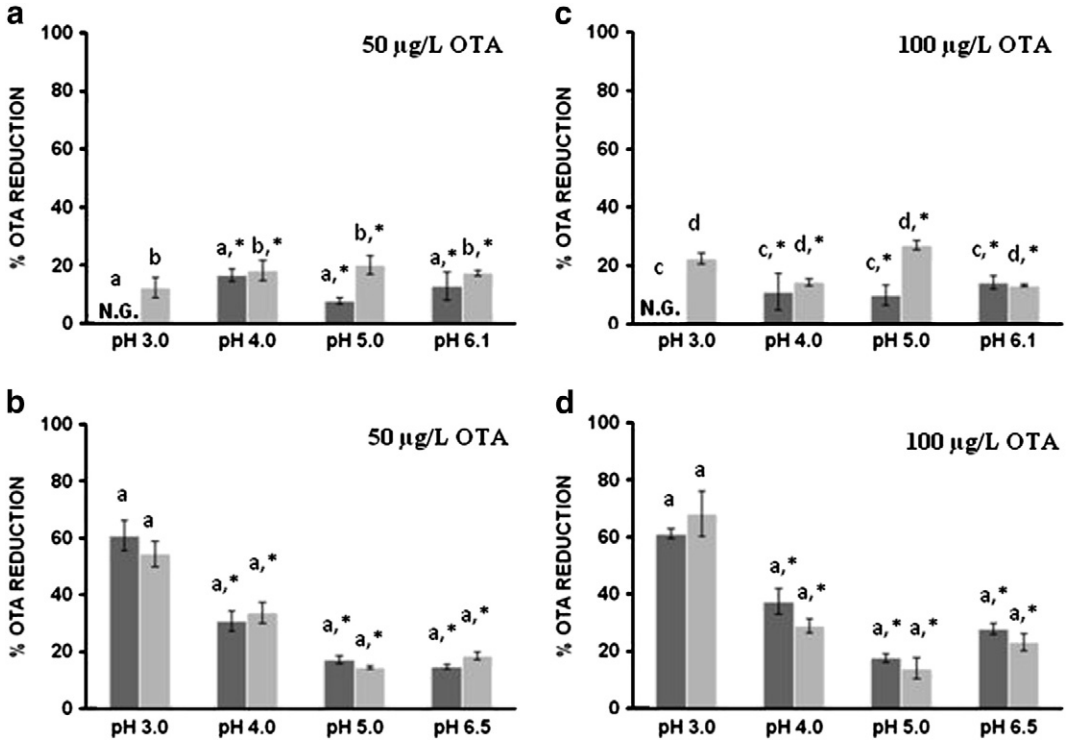

Fig. 4. Impact of cell density (10^3 cfu/mL — dark gray bars; 10^7 cfu/mL — light gray bars) of composites comprised of the: (a, c) 29 bacteria; and (b, d) 16 yeast strains, on OTA reduction (%), in response to pH (3.0, 4.0, 5.0, 6.1 or 6.5) and initial OTA concentration (50 and 100 μg/L), after incubation at 30 °C for 5 and 2 days respectively. Bars with a common letter for the same pH or initial OTA concentration represent not significantly different ($P>0.05$) OTA reduction. Star indicates statistical significance in comparison to the results obtained at pH 3.0 (*: $P<0.05$).N.G.: no growth was observed.

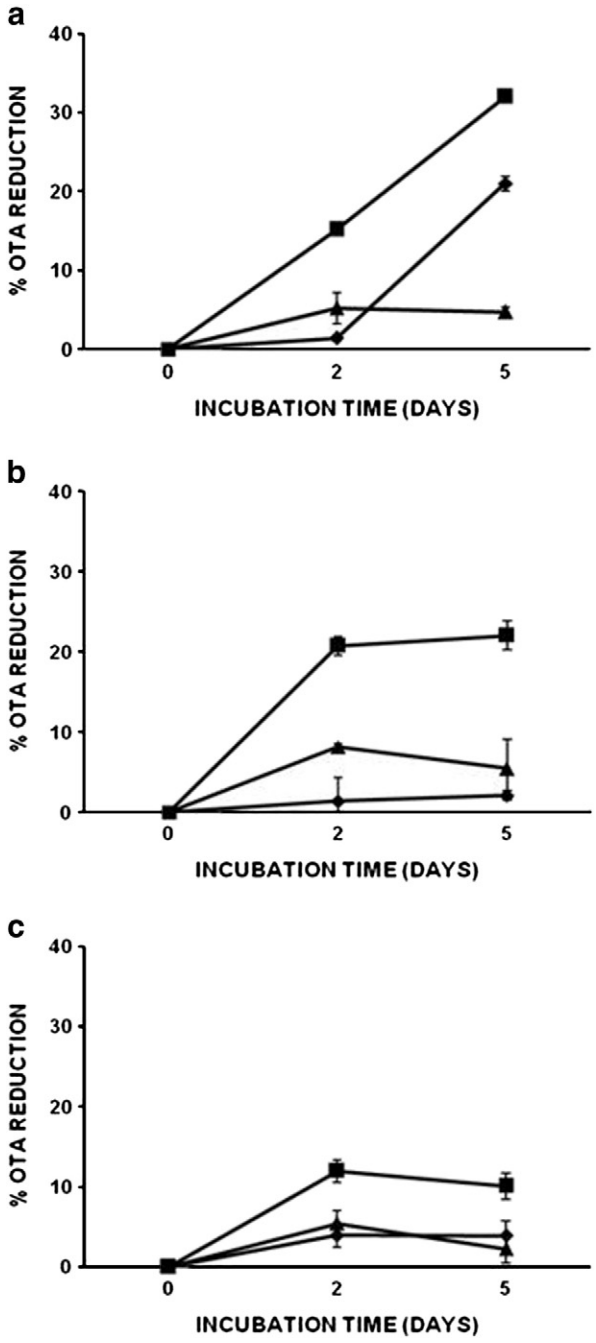

Fig. 5. Kinetics of % OTA reduction in: (a) grape juice, (b) red wine, and (c) beer fortified with toxin initial concentration of 100 μg/L, inoculated with 10^7 cfu/mL of 29BM (–▲–) and 16YM (–■–) composites and incubated at 25 °C for 5 days. Control samples (food products without the inoculated microorganisms) were also studied (–♦–).

1995). Differences on results between studies could be attributed to strain variability, nutrient content of culture media and pH. In our case, given that SGM is a culture medium "rich" in nutrients, a possible interpretation for the disagreement of our findings with those of previous reports may be its low pH (3.5), since the visible bacterial colonies on the agar plates were limited. This argument is also supported by the results of optical density measurements, in which all tested bacterial composites were inoculated (3log cfu/mL) on MRS broth with modified pH to 3, 4, 5, and 6.1. All bacterial composites had a minimum pH allowing growth around 3.5, except for *Lactobacilli* mixture, which could initiate growth at markedly higher pH values, around 4.5 (data not shown). Considering also that all tested bacterial strains, were isolated from food products of pH higher than 4.5–5.0, such a low pH value may cause additional stress. On the contrary, the adaptation of the tested yeast species was easier since the pH value of isolation origin was close to the one of SGM. Given this sensitivity of our studied bacterial composites to low pH values, their application on acidic foods in order to inhibit fungal growth, may be extremely restrictive. However, further assays of their inhibitory capacity against fungi at higher pH values and other culture media could be useful to resolve this issue.

In contrast to bacteria, almost all yeast composites showed a positive effect on *A. carbonarius* inhibition, at all studied assays. Moreover, our results showed that the highest inhibitory effect was obtained at a_w of 0.95, regardless of yeast composite and initial combination of cell/spore density. Differences on the degree of inhibitory ability of yeast composites against *A. carbonarius* were also observed, suggesting that each composite may have a different effect on *A. carbonarius* growth suppression. However, the positive effect of the applied yeast composites on fungal inhibition is consistent to previous reports, which studied the restrictive ability either of individual yeasts such as *Metschnikowia pulcherrima*, *Issatchenkia orientalis*, and *I. terricola* on *A. carbonarius* or of mixed cultures of *Pichia anomala*, *P. kluyveri* and *Hanseniaspora uvarum* on *A. ochraceus* growth (Bleve et al., 2006; Masoud and Høj Kaltoft, 2006). Our observations also suggest that by increasing the concentration of yeasts to 10^5 cfu/mL, the inhibition of *A. carbonarius* growth also increased compared to the initial cell density of 10^2 cfu/mL, indicating the possible existence of a minimum inoculum size of yeasts capable of achieving fungal growth inhibition. Several studies have reported that the antagonistic activity of yeasts against fungi may be also associated with competition for nutrients and space or adhesion of the cells to the fungal mycelium (Spadaro et al., 2002; Spadaro and Gullino, 2004). The present results suggest that the addition of yeast composites could be a reliable method for inhibition of *A. carbonarius* growth on food products of low pH (3.5) and a_w being in the range of 0.92–0.98, such as juices, musts, wines.

4.2. OTA reduction

The low ability of bacterial mixtures for OTA reduction at all examined combinations of pH and OTA initial concentration is consistent to the in vitro observations of del Prete et al. (2007), who studied the ability of fifteen strains belonging to five oenological LAB species to reduce OTA and reported decrease percentages of 8 to 28%. On the contrary, according to Mateo et al. (2010a), ten oenological strains of *Oenococcus oeni* reduced OTA *ca* 50–70%, cultured in MLO medium, whereas Fuchs et al. (2008) reported OTA reductions of >98% by *Lactobacillus acidophilus* in MRS broth after incubation for 4 h at 37 °C. Such variability in the results of different reports may be associated with strain diversity and origin of isolation, suggesting that the detection and selection of OTA detoxifying bacterial strains are a challenging task.

In contrast to other studies which suggest that the highest OTA reduction was observed at the lowest OTA initial concentration (Fuchs et al., 2008; Mateo et al., 2010a), our results indicated that the initial OTA concentration had no evident effect on % OTA decrease, regardless of pH and bacterial composite. Moreover, no growth was observed by all of the studied bacterial composites at pH 3.0, possibly due to the highly stressful effect of pH 3.0 on bacterial growth. Concerning the effect of cell density on OTA reduction, the high initial inoculum (10^7 cfu/mL) indicated higher ability for OTA reduction than 10^3 cfu/mL, consistently with the results of Fuchs et al. (2008). Considering the above, it may be suggested that the bacterial composites should be applied in combination with other detoxification methods i.e., physical or chemical, in order to increase their OTA reduction potential.

Contrary to the low detoxifying ability of bacterial composites, all the studied yeast composites were capable of reducing OTA, achieving a similar order of magnitude with the results of Cecchini et al. (2006) who studied OTA reduction during wine fermentation (up to

70%). Reduction in pH of the medium caused higher OTA reductions, a result which could be correlated with the wild nature of the studied yeasts, giving them the ability to grow at such low pH values. Some of the studied yeast species in this study e.g. *Zygosaccharomyces bailii, Kluyveromyces dobzhankii, Kazachstania hellenica*, were tested for the first time for their ability to reduce OTA and especially in composites, while others such as *S. cerevisiae* have been extensively studied, for their detoxifying potential on aflatoxins, OTA, and zearalenone (Santin et al., 2003; Yiannikouris et al., 2003). The high OTA reduction percentages suggest that the addition of the studied yeast composites could be an efficient method to reduce OTA in substrates belonging in wide range of pH (3.0–6.5). However, further study is necessary in order to elucidate the effect of more ecology parameters such as a_w and temperature, on OTA reduction.

Our results on beverages suggest that the % levels of OTA reduction were lower compared to those obtained in culture media (in vitro) at similar conditions of pH, OTA initial concentration and initial cell density. Such differences may be associated with the effect of intrinsic properties of food substrate i.e., a_w, microstructure, nutrients content, indigenous microflora which were not taken into account by the in vitro assays. However, the composite of 16YM showed higher percentages of OTA reduction compared to 29BM, regardless of the studied beverage, in agreement with the results from the in vitro experiments. No OTA reduction was observed in the controls of wine and beer samples, whereas control samples of grape juice showed remarkably high levels of OTA removal, indicating that some species belonging to endogenous microflora had also the ability to reduce the toxin. OTA reduction in beverages followed the order grape juice>red wine>beer. The lower pH of grape juice and wine (3.3–3.6) compared to beer (4.4) could possibly be responsible for these differences. The effect of the indigenous microflora should also be taken into account. Considering the observed ability of indigenous microflora for OTA reduction especially in grape juice, the inoculated mixture of 16 yeasts was most effective on wine. Although the latter effect could possibly be eliminated by conducting the experiment on sterile beverages, this does not constitute a realistic application. Similar to our results, Bejaoui et al. (2004) indicated that individual strains of *S. bayanus* and *S. cerevisiae* were also able to decompose OTA in synthetic and natural grape juice. Moreover, the limited variation of a_w and pH on the inoculated beverages during storage, indicated low spoilage activity of the tested foodstuff, an observation that was further supported by the maintenance of a satisfactory macroscopic appearance (color, turbidity, odor) of products, suggesting that these species could be used as protective cultures. The occurrence and metabolic properties of LAB species such as *P. pentosaceus, Lactobacillus sp.* and yeasts e.g. *S. cerevisiae* on the surface of malt barley and derived products may serve special functions, which have impact on processes such as improved quality, safety and flavor in the brewing industry (Booysen et al., 2002; Legras et al., 2007). However, despite the promising results of OTA reduction by the cultures tested in beverages, there is a clear need for optimization of the method in order to achieve the highest possible OTA reduction without altering the sensory properties of the product (protective cultures). Evaluating potential combinations of inoculation levels, culture compositions and time-temperature scenarios could be the scope of future studies. Furthermore, several researches suggest the use of heat inactivated (dead) cells (Bejaoui et al., 2004). *O. oeni* is considered as an important microorganism during wine making (final stages) with effective detoxifying properties on OTA under low pH values; however in this research bacteria with limited published data e.g. *W. cibaria*, *Streptococcus salivarius* (del Prete et al., 2007; Mateo et al., 2010a,b) were chosen to be studied. Nevertheless, the application of *O. oeni* in mixed cultures could be a future objective, whereas several of our species may be suggested for a detoxifying role during fermentation or malting. Therefore future work could involve mixing cultures during these procedures in order to evaluate any possibility for higher OTA reductions (Cecchini et al., 2006; Meca et al., 2010). Although the percentages of OTA reduction in beverages were significantly lower than those obtained in culture media, our findings could still be useful for the development of strategies to control OTA levels in certain foods.

Although there is no clear explanation about the mechanism of OTA detoxification when toxigenic mold and other microorganisms are co-cultured, the main theories are associated with the absorption of OTA to cell surface or degradation of OTA to less toxic compounds such as ochratoxin α. In our study, the use of immunoaffinity columns before the HPLC analysis makes it difficult to extract a safe conclusion about OTA detoxification mechanism. These columns are strictly specialized for OTA, eliminating by this way any prospect for detection of other degradation products. However, several studies suggest the use of bacteria such as *Streptococcus*, *Lactobacillus* and *Bacillus* or non toxic strains of *A. niger* as sources of carboxypeptidase A, the main cause of OTA degradation (Fuchs et al., 2008; Peteri et al., 2007; Varga et al., 2000). Other researchers suggest that enzymatic degradation of OTA can be also succeeded by lipases or metalloenzyme (Abrunhosa and Venâncio, 2007; Stander et al., 2000). Concerning the mechanism for OTA reduction, several studies support the ability of microorganisms to strongly bind the toxin on the external or even in the internal part of their cell wall (Meca et al., 2010; Ringot et al., 2005). Specifically, compounds such as proteins, polysaccharides, and lipids, are capable to form easily and accessible sites for adsorption (Huwing et al., 2001; Santos et al., 2000). Regarding yeasts as biocontrol agents, mannoproteins seem to have an important role in OTA adsorption by controlling the cell wall porosity (De Nobel et al., 1990; Nunez et al., 2008). Moreover, Piotrowska and Zakowska (2005), studied the reduction dynamics of OTA by LAB strains and suggested that elimination of the toxin was related to a binding mechanism to bacterial biomass. Taking into account all the above existing reports, and given the restrictions occurred by using immunoaffinity columns, further study is necessary to fully clarify the mechanisms that took place in our study and were responsible for OTA detoxification. This clarification could be the scientific basis in order to develop a molecular approach for OTA detoxification.

Overall, the results of the present study revealed that the highest percentages of *A. carbonarius* inhibition and OTA reduction were obtained by yeast composites compared to bacteria, in liquid laboratory media and beverages such as grape juice, wine, and beer. The results also indicated that factors such as a_w, pH, initial OTA concentration and initial cell density may significantly affect fungal suppression and OTA reduction, suggesting that these parameters should be considered when decontamination or a detoxification technique is applied. Considering the above, further study is necessary to enforce our results by incorporating the effect of more ecology parameters such as a_w, microstructure, nutrients which characterize a food substrate. Moreover, the mechanisms which are involved to competitive activity of those yeasts against *A. carbonarius* and detoxification of OTA need to be clarified. Such information could be a useful tool for controlling the risks of *A. carbonarius* presence and OTA production, ensuring by this way food safety and consequently consumer health.

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