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Lactic acid bacteria against post-harvest moulds and ochratoxin A isolated from stored wheat

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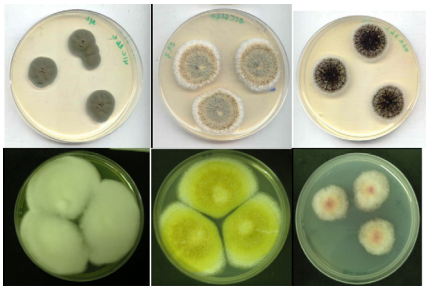
HIGHLIGHTS

- *Lactobacillus plantarum* exhibited a large antifungal spectrum.
- Lactic bacteria showed high inhibitory effect against ochratoxigenic fungal strain.
- Temperature, pH and bacterial biomass has a significant effect on fungal inhibition.
- Temperature, pH and bacterial biomass has a significant effect on OTA production.
- Selected LAB may be exploited as a potential OTA detoxifying agent.

GRAPHICAL ABSTRACT



Mould strains isolated from stored durum wheat.

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ABSTRACT

A total of 54 lactic acid bacteria (LAB) were isolated from stored wheat samples sourced from grain silos in North Tunisia. Fifteen representative isolates were identified by 16S rDNA sequencing as *Pediococcus pentosaceus*, *Lactobacillus plantarum*, *Lactobacillus graminis*, *Lactobacillus coryniformis* and *Weissella cibaria*. These isolates were screened for antifungal activity in dual culture agar plate assay against eight post-harvest moulds (*Penicillium expansum*, *Penicillium chrysogenum*, *Penicillium glabrum*, *Aspergillus flavus*, *Aspergillus niger*, *Aspergillus carbonarius*, *Fusarium graminearum* and *Alternaria alternata*). All LAB showed inhibitory activity against moulds, especially strains of *L. plantarum* which exhibited a large antifungal spectrum. Moreover, LAB species such as *L. plantarum* LabN10, *L. graminis* LabN11 and *P. pentosaceus* LabN12 showed high inhibitory effects against the ochratoxigenic strain *A. carbonarius* ANC89. These LAB were also investigated for their ability to reduce *A. carbonarius* ANC89 biomass and its ochratoxin A (OTA) production on liquid medium at 28 and 37 °C and varied pH conditions. The results indicated that factors such as temperature, pH and bacterial biomass on mixed cultures, has a significant effect on fungal inhibition and OTA production. High percentage of OTA reduction was obtained by *L. plantarum* and *L. graminis* (>97%) followed by *P. pentosaceus* (>81.5%). These findings suggest that in addition to *L. plantarum*, *L. graminis* and *P. pentosaceus* strains may be exploited as a potential OTA detoxifying agent to protect humans and animals health against this toxic metabolite.

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1. Introduction

Eukaryotic and prokaryotic organisms like moulds, yeasts and bacteria are natural contaminating microorganisms of cereal grains. Nevertheless, some filamentous fungi may develop at different phases of seeds handling especially when agricultural practices, storage conditions and processing stage are not well controlled. These moulds can cause irreversible damages and losses of quality product as well as the production of toxic metabolites in grain.

Ochratoxin A (OTA) is a fungal secondary metabolite produced by species belonging to *Aspergillus* and *Penicillium* genera, mainly *Aspergillus niger*, *A. ochraceus*, *Aspergillus carbonarius* and *Penicillium verrucosum*. This mycotoxin causes several negatives effects on animals and humans including nephrotoxic, teratogenic, embryotoxic, immunotoxic, genotoxic, and neurotoxic effects (JECFA, 2001; Wangikar et al., 2005). Moreover OTA is considered to be a possible human carcinogen and is classified as Group 2B by the International Agency of Research on Cancer (IARC, 2002). Ochratoxin A has been found in human food and animal feed like cereals, wine, cocoa, dried vine fruits, olives, coffee, beer, and spices (JECFA, 2001; Birzele et al., 2000; Fazekas et al., 2002; Roussos et al., 2006). Thus, their production in such commodities can be influenced by several factors, including temperature, water activity, pH, nutrients availability and competitive growth of other microorganisms. The contamination of food by mycotoxins can represent a direct source of human exposure by direct consumption or an indirect source through the consumption of derived products from animals fed with contaminated feed.

Due to the importance of wheat in the human diet and considering the impact of this mycotoxin on health, preventive measures for the reduction or the elimination of fungal growth and mycotoxins have received much attention in recent years. Safe strategies for this purpose were investigated by the use of microorganisms such as yeasts, filamentous fungi and bacteria as antimicrobial and mycotoxins detoxification agents (Sharma et al., 2009). In fact, the requirements of biological products with low impact on the environment, on human health and with ability to bind such toxic metabolites take increasing attention. The use of lactic acid bacteria (LAB) which are generally recognized as safe have received significant attention as a novel approach to the control of pathogens in foods by the production of antimicrobial compounds (Klaenhammer, 1993; Settani et al., 2005; Gerez et al., 2013) but only few studies have been investigated for the LAB efficiency in matter of OTA removal from foods (Skrinjar et al., 1996; Turbic et al., 2002; Piotrowska and Zakowska, 2005). Studies about this topic are necessary since wheat is the most important cereal crop in many countries including Tunisia. In this study, a series of bacterio-fungal culture experiments were carried out to: (i) analyze the development states of co-occurred fungus (*A. carbonarius*) and LAB under different temperatures and pH conditions. (ii) Analyze negative aspects of bacterial effects on fungal OTA production and biomass formation. For that, isolated LAB from stored durum wheat were initially identified. These investigations were made for the first time for *Lactobacillus graminis* and *Pediococcus pentosaceus*.

2. Materials and methods

2.1. Isolation of lactic acid bacteria

Lactic acid bacteria (LAB) were obtained from stored wheat samples sourced from grain silos in North Tunisia. According to the enrichment method described by Chen et al. (2005), durum wheat samples were inoculated in 10 ml MRS broth and incubated under anaerobic conditions for 2–3 days at 30 °C. After incubation, cultures obtained were serially diluted and spreaded in duplicate on the surface of MRS agar media usually used to isolate LAB associated to food matrices. In order to avoid fungal and yeast growth cycloheximide (0.01%) was added to the media. Petri dishes were anaerobically incubated for 2 days at 30 °C. Colonies arising on plates were picked on MRS media by successive sub-culturing for purification. The isolates were then characterized for morphological characteristics, Gram-positive, catalase-negative, non-mobile, cocci and rods colonies. Only isolates that showed negative reaction were maintained at −80 °C in glycerol stocks and subjected to further molecular identification.

2.2. DNA extraction and molecular identification of LAB

Genomic DNA was extracted according to CTAB/NaCl method described by Wilson (1987) and modified by using lysozyme (1 mg/ml) for cell lysis. Bacterial strains were characterized genotypically by 16S rDNA gene PCR amplification using universal primer pair s-d-bact-0008-a-S-20/s-d-bact-1495-a-A20 according to Daffonchio et al. (1998). PCR was performed on a thermocycler (BioRad) with initial denaturation at 94 °C for 3 min, 35 cycles of denaturation at 94 °C (45 s), annealing at 55 °C (1 mn), elongation at 72 °C for 2 min and a final extension at 72 °C for 10 min. PCR products were separated by electrophoresis through 1.5% (w/v) agarose gel containing ethidium bromide (0.5 mg/ml) and the DNA was detected by UV trans-illumination (UV spectrophotometer). 16S rDNA PCR amplicons were purified with Exonuclease-I and Shrimp Alkaline Phosphatase (Exo-Sap, Fermentas, Life Sciences). DNA sequencing was performed in an automated capillary DNA sequencer (Applied Biosystems 3130XL) using a Big Dye Terminator cycle sequencing Kit V3.1 (Applied Biosystems). Identification to the closet relative taxa of the isolates was achieved using BLAST analysis tool in the GenBank DNA database (www.ncbi.nih.gov). Phylogenetic analysis of 16S rRNA gene sequences was conducted with MEGA-5 software (Tamura et al., 2011). Phylogenetic tree was constructed by using neighbor-joining method (Saitou and Nei, 1987).

2.3. Nucleotide sequence accession numbers

The sequences of the 16S rDNA gene of LAB isolates have been submitted to the GenBank databases under accession numbers from KF554129 to KF554141.

2.4. Screening for antifungal activities against post-harvest moulds

2.4.1. Fungal strains and growth conditions

A total of eight post-harvest moulds were isolated from stored durum wheat in previous studies (Belkacem-Hanfi et al., 2013). Fungal isolates belonging to four genera of *Penicillium* (*Penicillium expansum* ANP234, *Penicillium chrysogenum* ANP67 and *Penicillium glabrum* ANP521), *Aspergillus* (*Aspergillus flavus* AFF94, *A. niger* ANN131 and *A. carbonarius* ANC89), *Fusarium* (*Fusarium graminearum* ANF14) and *Alternaria* (*Alternaria alternata* ANA212) were used for next antifungal experiment. Among these species, *A. carbonarius* ANC89 was selected as OTA producer reference strain. Spore suspensions of each fungal strain were prepared then grown on PDA medium for 7 days at 28 °C in 250 ml flask. In a next step, 100 ml of sterilized distilled water supplemented with Tween80 (0.01%) were added. Finally, spores were counted with Malassez-cell and suspensions were adjusted to about 10^5 spores/ml.

2.4.2. Antifungal growth activities of LAB isolates in dual culture

All LAB isolates were tested for their antifungal activities against post-harvest moulds using dual culture technique as described by Whipps (1987) with little modification: culture medium used for the test was MRS modified medium containing for

1 L: peptone 10 g, beef extract 10 g, yeast extract 5 g, glucose 20 g, K_2HPO_4 2 g, ammonium acetate 2 g, $MgSO_4$ 0.2 g, $MnSO_4$ 0.05 g, Tween80 1 mL and Agar 15 g. After agar solidification, 10 µl of each fungal spore suspensions (10^5 spores/ml) were inoculated in the center of Petri dish then each LAB isolate was inoculated at 20 mm away from fungal suspension. After incubation for 5–7 days at 28 °C, the plates were examined for inhibition zones. Fungal Petri plate without bacterial inoculation served as control. Growth inhibition of moulds was determined after the end of incubation according to the equation:

$$\% \text{ Inhibition} = [1 - (a/b)] \times 100$$

where; a = radial growth of fungal colony on medium with LAB, b = radial growth of fungal colony on medium without LAB.

2.5. Inhibition of A. carbonarius growth and ochratoxin A production

2.5.1. Preparation of LAB inoculum

Three isolated lactic acid bacteria (*Lactobacillus plantarum* LabN10; *L. graminis* LabN11 and *P. pentosaceus* LabN12) were grown on LTB medium according to Gizzarelli et al. (1993). Then, LAB were transferred to fresh broth and incubated for 48 h at 30 °C. After bacterial cells absorbance measuring at 600 nm, 100 µl of each LAB suspension (10^5 cells/ml) were used as inoculum for the current study.

2.5.2. Effect of LAB on A. carbonarius mycelium growth and OTA production

The experiments were performed by inoculating into 250 ml Erlenmeyer flasks containing 100 ml of LTB medium; 2×10^5 spores/ml of *A. carbonarius* strain (control 1); each of three LAB strains; LabN10, LabN11 and LabN12, 2×10^5 cells/ml (control 2); *A. carbonarius* and each of LAB strains, to obtain equal numbers of about 2×10^5 microorganisms/ml. Incubations were carried out at 28 and 37 °C for 7 days and the experiment was performed twice in duplicate. Initial pH of LTB broth was adjusted to 6.5 (before inoculation) and measured at the end of incubation with Hanna precision pH meter model pH 212 (Sigma–Aldrich). Enumeration of LAB as well as measures of *A. carbonarius* mycelium dry weight were carried out after 7 days of incubation. The counting of bacteria cells in LTB agar was performed with Malassez-cell. Effect of temperature on LAB development and *A. carbonarius* ANC89 growth was studied in mixed culture with selected LAB at 28 and 37 °C. Determination of OTA content on LTB medium produced by *A. carbonarius* ANC89 (control 1) and in mixed cultures with LAB was carried out.

2.5.3. Extraction of OTA

OTA extraction was made according to Petchkongkaew et al. (2008) with some modifications: Five milliliters of each culture were sampled at the end of experiment and centrifuged for 15 min at 12.000 rpm and 34 µl of 12 N HCl were added to the supernatant followed by 3.5 ml of chloroform. After agitation, the chloroform phase was recuperated, evaporated under nitrogen gas at 65 °C and dissolved in 1 ml of methanol. Then, extracts were filtered through 0.22 µm Millipore filter (Whatman, Germany) and analyzed by HPLC as described by Roussos et al. (2006).

2.5.4. Quantification of OTA

HPLC analysis was carried out with a C18 column (Waters RP; 150 mm; 4.6 mm) in which sample volumes of 20 µL were injected. Chromatographic separation was performed under isocratic conditions at a flow rate of 1 ml/min of the mobile phase which consisted of a mixture of acetonitrile/water/acetic acid (50:50:2 v/v/v). OTA was detected using a UV-detector (Waters 490E) at 335 nm and OTA retention time was approximately 13 min. The percentage of OTA removed from LTB broth was calculated by the following formula:

$$\% \text{ OTA} = 100 \times [1 - (\text{OTA peak area of sample} / \text{OTA peak area of control})]$$

2.5.5. Statistical analysis

Using multivariate analyses, we tried to highlight relationships between fungal–bacteria interactions and input and output culture parameters (at 28 and 37 °C). For that, a standardized principal component analysis (PCA) was applied on the table containing 8 types of cell cultures in rows and 5 input/output measured parameters. PCA was carried out by using the JMP statistical software (SAS, 2008).

PCA is a multivariate analysis which decomposes the structure of a high-dimension system (dataset) into several one-dimension blocks with decreasing variability called principal components (PCs) (Jolliffe, 2002). PCs have particular spatial directions along which the whole information of the initial dataset is hierarchically decomposed into well-structured and cumulative variability parts: the first PC (P1) extracts the highest variability of the studied system followed and completed by the second PC (P2), and so on. This helps to understand step-by-step the complex organization of the whole system as well the trends which control it. The new directions defined by PCs attribute to the individuals (bacterial cultures) new coordinates the closeness and remoteness of which are indicative of different similar and different behaviors, respectively. These coordinates are calculated by linear combinations between the initial data and a serial of vectors (called eigenvectors) containing coefficients of the different variables. These coefficients have absolute values ($0 \leqslant x \leqslant 1$) which vary according to the role (weight) played by each variable in the multidimensional system. Graphical projections of these coefficients give the correlation circle in which the different trends of variables are represented by vectors with different directions. Each vector gives a balance trend of a variable (a culture parameter) taking into account the effects of all the other variables in the system: in the correlation circle, acute, obtuse and right angles between variables' vectors mean positive, negative and not-significant trends, respectively. From the factorial plot of two PCs (e.g. P2 vs P1), projections of points in different quadrants (upper right, upper left, lower right, lower left) help to identify different groups having distinct behaviors in the studied population. Such groups are associated to subsystems of variables which are identified in the same quadrants given by the correlation circle.

In this work, the factorial plots associated to the first two PCs were used to analyze trends between the five culture parameters. At the same time, the factorial coordinates of the eight cultures along the first two PCs were projected to analyze their response traits the ones relatively to the others under the effects of different culture parameters (consulted from the correlation circle). To make easy the identification and interpretation, the eight cultures were encoded by three terms including temperature (28 or 37 °C) followed by "Ac" for *A.carbonarius* then bacteria code (10, 11, 12 for LabN10, LabN11, LabN12, respectively). The two control fungus cultures (without bacteria) were encoded by corresponding temperature followed by "Ac", and were treated as supplementary elements. Moreover, the five measured parameters were encoded by NB, pH, DW, BioRed and OTAred designating the number of LAB, pH, dry weight, fungal biomass reduction and fungal OTA reduction, respectively.

3. Results

3.1. Isolation of LAB and molecular identification

Upon enrichment in selective broth 54 colonies were picked and purified. A total of 15 strains were selected on the basis of their

unique morphology and identified by partial 16S rRNA gene sequencing. According to the comparison of 16S rDNA sequences - with those available in the GenBank, all strains were affiliated to LAB species with sequence similarity > 97% (Table 1). In particular, identification of selected LAB from stored durum wheat was assigned to three genera: *Lactobacillus* (*L. plantarum*, *L. graminis*, *L. coryniformis*), *Pediococcus* (*P. pentosaceus*) and *Weissella* (*Weissella cibaria*). Phylogenetic tree highlighted five different genotypic groups that are consistent with the identified species (Fig. 1).

3.2. Comparative antifungal activity of LAB

All selected strains were screened for inhibitory activity towards 8 post-harvest moulds belonging to *Penicillium*, *Aspergillus*, *Alternaria* and *Fusarium* genera using dual culture method. Varied percentages of bacterial mediated inhibition of mould production were detected against the post-harvest moulds (Table 1). All LAB strains were able to produce apical reduction of two or more moulds on modified MRS medium. All LAB strains inhibited *A. alternata* and *P. chrysogenum*. Also, three LAB strains (*L. plantarum* LabN9, LabN10 and LabN14) have high antifungal activity against all fungal strains and were the only effective against *A. niger*, *L plantarum* (LabN10 and LabN15), *L. graminis* LabN11 and *P. pentosaceus* LabN12 showed a significant radial growth reduction against *A. carbonarius* ANC89 (60%, 60%, 60% and 55.5%, respectively). In addition, the majority of LAB isolates highly inhibited *A. flavus*, *F. graminearum* and *P. expansum* with % inhibition $\geqslant$ 51.1%, 47.7% and 47.7%, respectively. A similar high percentage of fungal inhibition for *P. expansum* was obtained by *L. plantarum* LabN7 and *L. graminis* LabN11 (56.8%). Notably, almost all isolated LAB exhibited a lower inhibitory effect for *P. glabrum* ($\leqslant$20%). Among LAB with higher antifungal activity against *A. carbonarius*, three LAB species *L. plantarum* LabN10, *L. graminis* LabN11 and *P. pentosaceus* LabN12 were selected and tested for their ability to inhibit *A. carbonarius* ANC89 growth and its OTA production in liquid media.

Finally, global differences between average growth inhibitions due to bacterial species were tested by ANOVA by grouping all the strains of a same species (viz. five bacterial species; Table 1). A significant difference between the five average growth inhibitions was found with a p-value = 0.0515 (α-risk $\leqslant$ 6%). Then, a Student test was carried out between the five bacterial species (compared two-by-two) and revealed a high significant difference between the radial growth inhibitions due to *L. plantarum* and *P. pentosaceus* (p-value = 0.003); this high significance was followed by that between *L. graminis* and *P. pentosaceus* (p-value = 0.084). All the other p-values were higher than 0.25. Behavioral differences between these three most distant bacterial species were invested by PCA by considering their representative strains (LabN10, 11, 12). These strains distinguished from other ones by their stronger growth inhibition patterns especially against *A. carbonarius*.

3.3. Variation of A. carbonarius growth and ochratoxin A production and influence of incubation temperature and pH

The PCA applied on the set of fungal–bacterial cultures gave two principal components (PCs) (P1, P2) which explained 46.7% and 28.3% of the total variance, respectively, i.e. 75% in all.

In factorial plot of individuals, the first PC (P1) clearly separated two temperature-dependent subsets, viz.: 37Ac12, 37Ac10 and 37Ac11 (right part) separated from 28Ac10 and 28Ac11 (left part) (Fig. 2a). For a same bacterial species, cultures at 37 °C generally showed higher pH, NB and DW vs lower OTAred than those performed at 28 °C. Such a topology revealed a significant effect of temperature on the general states of different fungus–bacteria systems. The correlation circle showed that pH and NB group along P1

Table 1
Antifungal activity of isolated LAB against 8 post-harvest moulds and molecular identification.

Strains	Radial growth inhibition (%)								Molecular identification	
	A. alternata ANA 212	*P. expansum ANP 234*	*P. chrysogenum ANP 67*	*P. glabrum ANP 521*	*F. graminearum ANF 14*	*As. niger ANN 131*	*As. carbonarius ANC 89*	*A flavus AFF 94*	Genera/species	% Similarity
LabN1	50	–	8.69	–	–	–	–	–	*Pediococcus pentosaceus*	99
LabN2	50	47.7	8.69	–	59	–	–	–	*Pediococcus pentosaceus*	99
LabN3	59	–	4.34	8	–	–	15.5	53.3	*Pediococcus pentosaceus*	97
LabN4	59	47.7	4.34	16	50	–	15.5	51.1	*Weissella cibaria*	97
LabN5	56.8	54.5	34.7	8	56.8	–	11.11	51.1	*Lactobacillus*	97
LabN6	56.8	52.2	4.34	20	56.8	–	11.11	53.3	*Lactobacillus graminis*	98
LabN7	56.8	56.8	21.7	20	56.8	–	–	51.1	*Lactobacillus plantarum*	97
LabN8	59	52.2	8.69	16	56.8	–	–	51.1	*Pediococcus pentosaceus*	99
LabN9	59	50	4.34	16	47.7	53.3	55.5	53.3	*Lactobacillus plantarum*	97
LabN10	59	50	8.69	20	54.5	53.3	60	51.1	*Lactobacillus plantarum*	97
LabN11	59	56.8	13.04	20	56.8	–	60	51.1	*Lactobacillus graminis*	98
LabN12	59	50	13.04	16	54.5	–	55.5	51.1	*Pediococcus pentosaceus*	99
LabN13	50	50	17.39	8	56.8	–	–	53.3	*Pediococcus pentosaceus*	97
LabN14	59	50	17.39	20	59	51.1	55	51.1	*Lactobacillus plantarum*	97
LabN15	54.5	50	8.69	12	56.8	–	60	–	*Lactobacillus plantarum*	97

–, no visible inhibition; *A.*, *Alternaria*; *P.*, *Penicillium*; *F.*, *Fusarium*; *As.*, *Aspergillus*.

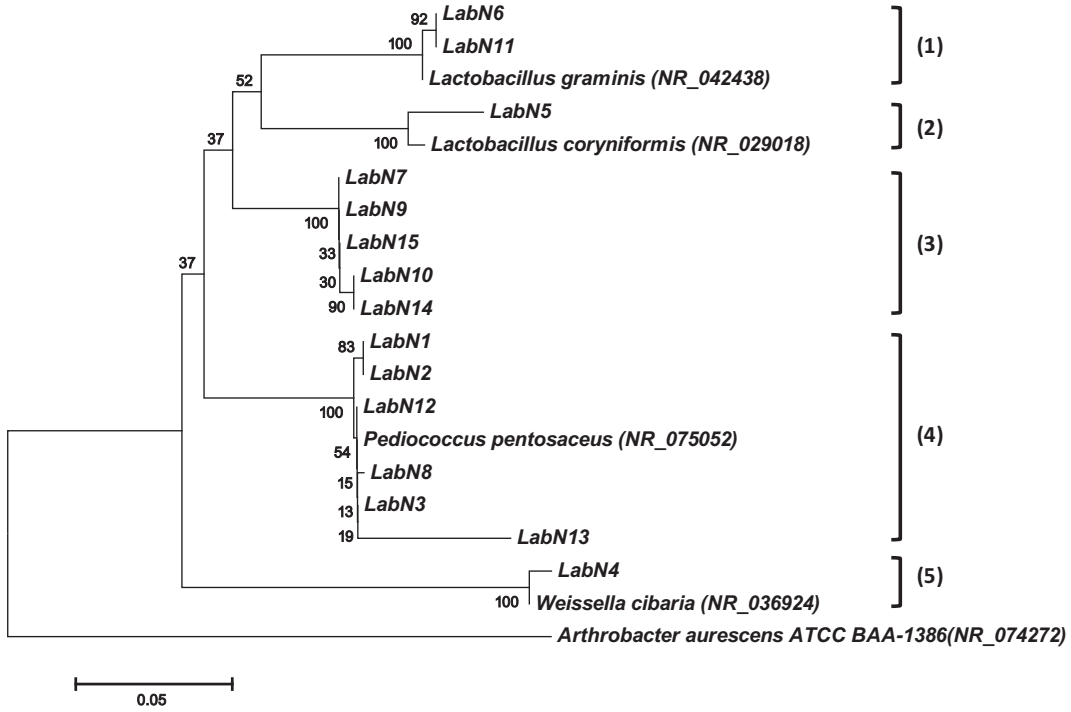

Fig. 1. Phylogenetic tree showing the relative position of lactic acid bacteria isolated from stored durum wheat based on 16S rDNA sequences, using the neighbor-joining method. *Arthrobacter aurescens* was used as an out group. Bootstrap values for a total of 1000 replicates are shown at the nodes of the tree. The scale bar corresponds to 0.05 units of the number of base substitutions per site.

(lower right quadrant) by opposing to OTAred (projected in upper left quadrant) (Fig. 2b). This topology combined with the projection of 37Ac12, 37Ac10 and 37Ac11 along the right part of P1 could be indicative of both higher bacterial development (NB) and lesser reduction of fungal OTA production under 37 °C and high pH (>6.5). This contrasting situation could be explained by particular culture conditions combining bacterial favorable temperature (37 °C) with fungal favorable pH (>6.5). The lower reduction of OTA in presence of higher bacterial development (under 37 °C and pH > 6.5) could reveal some hierarchical effect of pH on OTA production: the favorable pH to fungi would have been at the origin of lower OTA reduction despite the high bacterial development favored by 37 °C. The high significant effect of pH on fungal development could be also revealed by the projection of dry weight (DW) in the upper right quadrant indicating higher fungal development which opposites to OTA reduction (upper left quadrant). Inversely, opposite situation was observed along the left part of P1, viz. 28Ac10 and 28Ac11, where higher fungal OTA reduction occurred despite low NB and temperature (28 °C) (Table 2). Fungus-favoring conditions seemed to be countered by low pH (favorable for bacteria) which would be at the origin of negative stress on fungal OTA production (higher OTAred). This confirms the hierarchical role of pH on observed OTA levels in microbiological cultures. However, the relatively low temperature (28 °C) seemed to be responsible for reduction of bacterial growth (NB) (Fig. 2b) as well in 28Ac10 as in the two other cultures 28Ac11 and 28Ac12 (Fig. 2a).

Beyond pH and temperature, the variation of OTA revealed to be bacterial species-dependent: In the lower right quadrant, the culture system 37Ac12 showed extreme position both along P1 and P2 (Fig. 2a). In addition to its high pH and high bacterial development (NB), the culture 37Ac12 showed important reduction of fungal biomass (Biored) (lower part of P2) (Fig. 2b). Despite the relatively important fungal biomass reduction (BioRed), the culture 37Ac12 showed the lowest OTA reduction among all the cultures. Taking into account the fact that the other bacteria cultures carried out at the same temperature (37Ac10, 37Ac11) did not project in the lower right quadrant, one could deduce that the species LabN12 would be the less active against OTA production (Table 2). This observation is confirmed by the fact that the culture 28Ac12 (bacteria species LabN12 at 28 °C) showed lower OTA reduction compared to the other bacteria cultures (28Ac10, 28Ac11) performed at the same temperature (28 °C).

Along the upper part of P2, the projection of DW and OTAred could be indicative of some unfavorable culture systems in which OTA reduction is important despite a high fungal DW (Fig. 2b). This situation concerned the culture systems 28Ac11 and 37Ac10 which showed the second and third highest OTA reduction after 28Ac10 (Fig. 2a) (Table 2). Taking into account the similar OTA states of 28Ac10 and 37Ac10, the extreme variation of fungal dry weight (from minimal in 28Ac10 to maximal in 37Ac10) cannot be considered to influence OTA levels. However, the shared OTA reduction in 28Ac10 and 37Ac10 could be compatible with their common bacteria species (LabN10) characterizing these two culture systems. This could be indicative of (i) bacterial species-dependent stress or (ii) some specific fungus–bacteria interactions resulting in OTA reduction: the bacteria species LabN10 seemed to be responsible for higher OTA reduction (compared to LabN11 and LabN12) whatever pH and temperature conditions (Table 2).

However, in the absence of bacteria, the fungus species *A. carbonarius* seemed to be influenced mainly by high pH values independent of the temperature range 28–37 °C: this can be checked by the projection of the two control fungus culture points (28Ac and 37Ac; supplementary elements) in the lower right quadrant (Fig. 2c). From this, we demonstrated the importance of temperature range (28–37 °C) as a significant modulatory factor in fungus–bacteria interaction systems.

4. Discussion

Lactic acid bacteria are used as biopreservatives and are considered to be active against a large range of food contaminant microorganism (e.g. moulds) by the production of antimicrobial

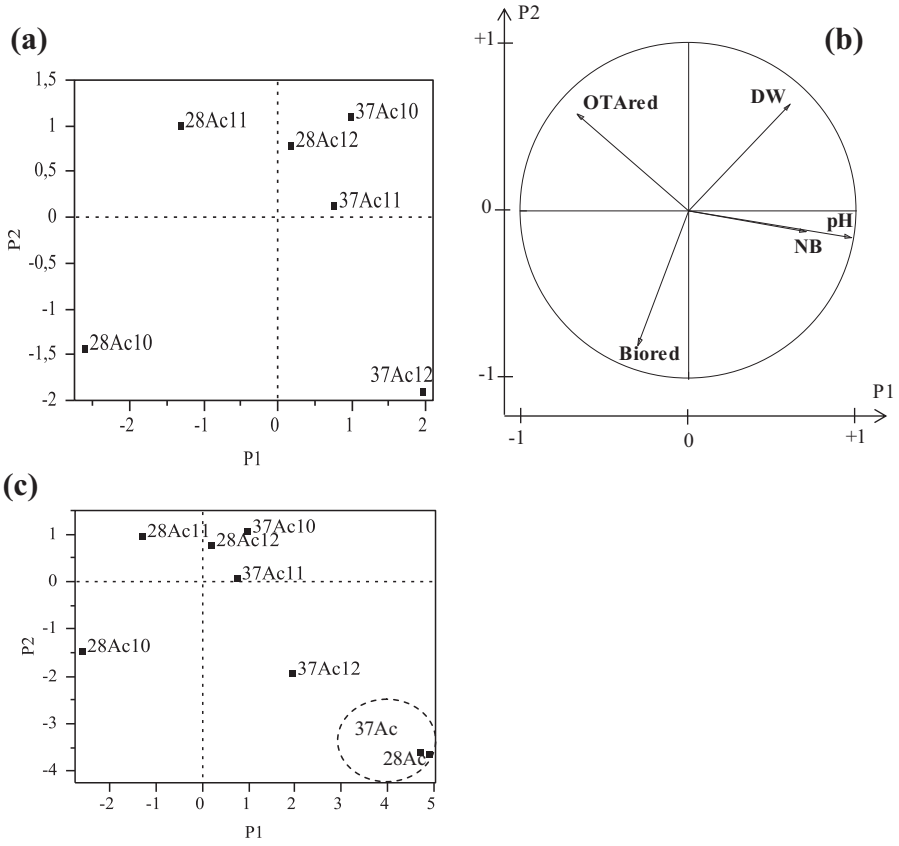

Fig. 2. Factorial plots representing the two first principal components P1, P2 of standardized PCA applied on dataset of 8 microbiological cultures characterized by 5 input/output parameters. (a) Individuals' factorial plot without control cultures. (b) Correlation circle showing trends between different culture parameters. (c) Individuals' factorial plot with control cultures (supplementary elements). Control cultures are fungal culture in absence of bacteria. Legend: NB: bacterial counts; DW: fungal dry weight; OTAred: OTA reduction; BioRed: biomass reduction; pH: potential of hydrogen. The individuals represented microbiological cultures which were encoded by temperature (28 or 37 °C) followed by Ac (for the fungus *Aspergillus carbonarius*) then by the bacteria label (10, 11, 12 for *Lactobacillus plantarum* LabN10, *L. graminis* LabN11 and *Pediococcus pentosaceus* LabN12, respectively).

Table 2
Number of bacteria, pH, *Aspergillus carbonarius* biomass and OTA reduction in mixed cultures after 7 days of incubation at 28 and 37 °C.

Cultures	NB	pH	DW	BioRed	OTAred
28Ac	0	7.3	621	0	0
28Ac10	7.3	4.8	376	39.5	99
28Ac11	7.5	4.9	496	20.2	98.9
28Ac12	7.3	5.9	517	16.8	92.9
37Ac	0	7.1	622	0	0
37Ac10	8.4	6	658	26.2	97.9
37Ac11	9.5	5.9	481	22.7	97.6
37Ac12	8.7	6.6	492	31.6	81.5

NB: bacterial counts; BioRed: fungal biomass reduction; DW: fungal dry weight; OTAred: OTA reduction; Ac: *Aspergillus carbonarius* (control); Ac10: *Aspergillus carbonarius* with *Lactobacillus plantarum* in mixed culture; Ac11: *Aspergillus carbonarius* with *Lactobacillus graminis* in mixed culture; Ac12: *Aspergillus carbonarius* with *Pediococcus pentosaceus* in mixed culture.

substances like organic acids and bacteriocins. In this study, lactic acid bacteria were identified on the basis of 16S rRNA gene sequencing and phenotypic characterization. According to our results, all strains were members of mainly lactic acid bacterial genera. Molecular identification of LAB strains revealed a major presence of *Pediococcus* and *Lactobacillus* genera in analyzed stored wheat. We observed that *Pediococcus* genus was represented by *P. pentosaceus* as the most frequently isolated LAB. Among the genus *Lactobacillus*, *L. plantarum* was found to be the most common species in our isolates followed by *L. graminis* and *L. coryniformis*. *W. cibaria* was represented at low frequency compared to the other species. Previous works showed strong LAB associations with plant material or cereal products: *P. pentosaceus* is normally found in fermented vegetables (Jonganurakkun et al., 2008; Yang and Chang, 2010) while *L. plantarum*, *L. graminis*, *L. coryniformis* and *W. cibaria* are commonly isolated from grass silage, wheat grains and fermented wheat products like buckwheat and sourdough (Moroni et al., 2011; Hancioglu and Karapinar, 1997; Magnusson and Schnürer, 2001; Corsetti et al., 2007; Kivanc et al., 2011).

In order to select bacterial strains with antifungal activity against filamentous fungi, we tested identified LAB against eight post-harvest moulds. All of them revealed ability to inhibit mycelium growth with various amounts. Almost all strains showed a strong antifungal activity against *A. alternata*, *P. expansum*, *F. graminearum* and *A. flavus*. Moreover, the LAB species *L. plantarum*, *L. graminis* and *P. pentosaceus* showed inhibitory effects against fungal strains among which *A. carbonarius* was the most sensitive. However, *A. niger* mycelium growth was inhibited by only three LAB strains belonging to *L. plantarum* species. In our experiments, *L. plantarum* revealed to be characterized by a large antifungal activity spectrum. Previous works showed this large activity against several moulds (Lavermicocca et al., 2000; Laitila et al., 2002; Rouse et al., 2008). Recently, an antifungal strain *L. plantarum* CRL 778 isolated from sourdough was described by its ability to inhibit *A. niger* and *F. graminearum* mycelium growth (Dal Bello et al., 2007). In addition, Djossou et al. (2011) confirmed *L. plantarum* antifungal activity of strains isolated from coffee against two *A. carbonarius* ochratoxigenic strains.

It is known that certain LAB strains are able to detoxify mycotoxins such as AFB1 (Pierides et al., 2000; Zinedine et al., 2004), *Fusarium* toxins (El-Nezami et al., 2002), patulin (Fuchs et al., 2008) and fumonisin B1 (Pizzolitto et al., 2012) but little investigations were conducted for the degradation of ochratoxin A (del Prete et al., 2007; Fuchs et al., 2008). In the present study, *L. plantarum*, *L. graminis* and *P. pentosaceus* were investigated for their ability to inhibit *A. carbonarius* ANC89 growth and its OTA production (in liquid medium). Our results showed that OTA level in bacterio-fungal cultures were subject to several physical–chemical and biological factors including temperature, pH, fungal resistance and bacteria species-dependent stress. Relatively higher reductions of OTA were observed in presence of *L. plantarum* LabN10 and *L. graminis* LabN11 (>97% OTA reduction) followed by *P. pentosaceus* LabN12 strain (81%) under different temperatures (28 or 37 °C) and pH (4.8 or 6). These results firstly highlight the importance of good selection of LAB for mould growth control and OTA level reduction in potentially exposed foods as wheat. For the three strains, better results were observed at 28 °C than at 37 °C. Its known that the temperature is a significant factor capable of stimulating or inhibiting OTA production by fungi, but little is known about the effect of pH on OTA production by *A. carbonarius* strains (Palacios-Cabrera et al., 2005; Bouras et al., 2009; Kapetanakou et al., 2009).

In our case, we observe that the inhibition of growth of *A. carbonarius* and OTA reduction by the three LAB is due to pH variation on media and might be a result of fermentative compounds accumulation. In fact, organic acids diffuse through the membrane of the target organisms in their hydrophobic undissociated form to reduce cytoplasmic pH and stop metabolic activities (Axelsson, 1990; Piard and Desmazeaud, 1991). Also, it is hypothesized that organic acids neutralize electrochemical potential of plasmic membrane and increase its permeability leading to bacteriostasis and eventually to the death of susceptible organisms (Batish et al., 1997). This property was demonstrated for acetic and propionic acids (Eklund, 1989). Acid compounds productions could be partly responsible for higher OTA reduction by *L. plantarum* LabN10 strain via their direct inhibition effect on *A. carbonarius* growth. This hypothesis could be compatible with the extreme state observed in the culture 28Ac10 where fungal biomass and OTA showed maximal reductions in presence of the lowest pH values. Moreover, the extreme antifungal activity of the culture 28Ac10 could have been favored by temperature (28 °C), an essential factor that modulates LAB growth and significantly affect the production levels of antifungal metabolites (Batish et al., 1997). Studies on *L. plantarum* CUK 501 demonstrated that antifungal activity was maximal at 30 °C when the culture was at the end of its logarithmic phase (Sathe et al., 2007). This shows some interactive effects of temperature depending on biological material and states.

Curiously, among our microbial cultures, those with highest bacterial counts did not show the highest OTA reductions: apart from the effect of LAB species variation, this could be partly explained by some reversible antifungal mechanism in LAB which are independent from cell viability. These mechanisms include binding of mycotoxins to LAB peptidoglycan wall and/or surface proteins (Piotrowska and Zakowska, 2005; Niderkorn et al., 2009). Several works showed that removal of OTA from medium consisted of partly reversible process in which the mycotoxin was released back into medium (Piotrowska and Zakowska, 2005; Del Prete et al., 2007; Fuchs et al., 2008). These results were consistent with another study on *Lactobacillus rhamnosus* GG showing ability of LAB to eliminate 80% of aflatoxin B from the medium exclusively through physical binding by the cell components (El-Nezami et al., 2002). Taking into account such reversible process, *P. pentosaceus* LabN12 strain which gave lower OTA reduction could release more rapidly bound OTA. Another hypothesis is that there could be less resistance against fungal competition in LabN12 than the two other strains *L. plantarum* LabN10 and *L. graminis* LabN11.

Lower resistance of LAB could be linked to several intrinsic and extrinsic factors. Hydrogen peroxide (H_2O_2) is a highly active compound produced by most LAB in presence of oxygen and can significantly reduce the growth of several undesirable micro-organisms (Kandler, 1983). However, LAB are unable to produce catalase for degradation (detoxification) of this lipid membrane and cellular protein oxidizing compound (Lindgren and Dobrogosz, 1990). Moreover, non-lethal levels of hydrogen peroxide may modulate fungal catalase and superoxide dismutase, two major enzyme involved in detoxification (Angelova et al., 2005). Comparative synthesis of such results led to conclude that OTA reduction consisted of a processes varying and depending on bacteria strains (Piotrowska and Zakowska, 2005).

In conclusion, the results of the present study revealed that the highest percentages of growth inhibition and OTA reduction in *A. carbonarius* was obtained by *L. plantarum* and *L. graminis* species. The results also indicated that factors such as temperature, pH and bacterial biomass on mixed cultures, have a significant effect on fungal inhibition and OTA production. For the first time we demonstrate that in addition to *L. plantarum*, *L. graminis* and *P. pentosaceus* were able to inhibit *A. carbonarius* growth and are potential OTA detoxifying strains with better activity for *L. graminis.* So, it will be interesting to investigate and to understand the mechanism induced by the reduction of fungal biomass and the OTA production by LAB and then to characterize their antifungal compounds. From our results, fungal proliferation control by these LAB strains can effectively improve the shelf-life of many stored products like wheat especially against ochratoxigenic strains and then reduce consumer health risks due to ochratoxin A exposure.

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