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ORIGINAL ARTICLE

Isolation of lactic acid bacteria from grape fruit: antifungal activities, probiotic properties, and in vitro detoxification of ochratoxin A

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Abstract

The aim of this study was to isolate lactic acid bacteria from two varieties of grape bunch cultivated in Tunisia (cardinal and red-globe) in order to assess their ability to inhibit the growth of their most widespread contaminant, *Aspergillus niger aggrégats* and *Aspergillus carbonarius*. Antifungal activity of 18 isolates was investigated using overlay technique; selected isolates were than identified using 16s rDNA sequence analysis. Isolates with antifungal activities were screened out and studied for their probiotic properties using in vitro tests (tolerance to simulated gastric jus, bile salts, hydrophobicity properties).The most efficient strain was also investigated for its ability to reduce the concentration of ochratoxin A (OTA) on liquid medium. Determination of OTA content in media was released using HPLC analysis. Selected strains (RG7B) (C11C) and (RG8A) showing a good antifungal activities against *Aspergillus niger aggrégats* and *Aspergillus carbonarius* were identified as *Pediococcus pentosaceus* and *Lactobacillus plantarum*, respectively. *Pediococcus pentosaceus* (RG7B) showed promising potential probiotic characteristics and had a high ability for OTA removal after 48 h of incubation in both MRS and PBS media. The OTA removal percentage was significantly higher in MRS than in PBS media (84 and 25%, respectively). This study provides evidence for the control of black *Aspergillus* growth in grape and OTA detoxifying by the use of autochthones LAB strains having antifungal effect and probiotic potential.

Keywords Lactic acid bacteria · Antifungal activities · Probiotic · Ochratoxin a

Introduction

Grapes and their derived products, wine in particular, are among the key components of the Mediterranean diet (Covarelli et al. 2015). Grapes are popular fruits in Mediterranean countries as they are consumed by a wide range of people. In Tunisia, the harvest of grape fruit, for the season 2014–2015, was 150,000 tons, representing an increase of 9% as regard the previous season (Communiqué of the Tunisian Ministry of Agriculture). However, grapes are exposed to many stress factors, from harvesting until storage and exposition in market. The major problem is contamination by spoilage fungi which cause numerous grape diseases and are responsible for qualitative and quantitative losses.

The grape microbial consortium is composed of highly diverse microorganisms, including yeasts, bacteria, and fungi (Rousseaux et al. 2014). Many studies have been interesting in yeasts and lactic bacteria, but there is also, in this environment, another microorganism responsible for the damage and deterioration of grape. Fungal diseases represent the most important threats to grapes and grape-derived products, often resulting in yield losses and severe quality reductions due to the accumulation of mycotoxins, secondary metabolites biosynthesized by some fungal species also present in the grape mycoflora (Covarelli et al. 2015).

Among these mycotoxins, Ochratoxin A (OTA) represents the most important candidate on grapes and their derived products. It is produced by molds belonging to several species of the genera *Aspergillus* and *Penicillium*. Ochratoxin A was classified in 1993 by the International Agency for Research on Cancer as a potential human

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carcinogen (IARC, 1993). It is also a potential nephrotoxin and hepatoxin with teratogenic, mutagenic, and immunosuppressive effects (Krogh, 1992, Creppy, 1998). According to different surveys, grape products from the Mediterranean regions of Southern Europe (Burdaspal and Legarda, 1999; Battilani et al. 2003; Belli et al. 2004a) and North Africa (Filali et al. 2001;Lasram et al., 2012) were the most contaminated by OTA. The contamination with OTA is essentially due to the growth of OTA-producing fungi on grapes during maturation. Black *Aspergillus* spp. were identified as the predominant OTA-producing molds present on grapes worldwide. Several studies showed that within this group, *Aspergillus carbonarius* was predominantly responsible for the production of OTA in grapes cultivated in South European countries (Cabanes et al. 2002; Bellí et al., 2004b; Serra et al., 2005; Battilani et al., 2006; Gómez et al., 2006; Tjamos et al., 2006), in Australia (Leong et al., 2004), and in Tunisia (Lasram et al., 2012). Of note, other black *Aspergillus* species, belonging to the *Aspergillus niger*, displayed lower OTA production. Therefore, to inhibit the contamination of grapes and derived products by mycotoxin, it is necessary to stop the development of responsible fungi such as *Aspergillus* ssp. As known, the most common food preservation strategies applied in the food industry involve chemical or physical techniques. However, these methods only reduce fungal infections but do not extend to elimination of contaminants. In addition, current consumer trends focus on high-quality, minimally processed green-label foods, thus driving the food industry towards a focus on natural preservation and stabilization approaches (Reis et al. 2012). To prevent the contamination by molds and production of mycotoxin, recent application of biopreservation agents has gained increasing interest. In particular, lactic acid bacteria are usually considered to be able to suppress the growth of other microorganisms including yeasts and molds. Due to their important history of application as starter cultures, especially in the food industry, lactic acid bacteria (LAB) have been intensively studied as bioprotective agents (Peyer et al. 2016). Recent research revealed that LAB can produce a range of antifungal substances including organic acids, proteinaceous compounds, and low-molecular-weight substances, e.g., phenyllactic acid, reuterin, cyclic peptides, 3-hydroxylated fatty acids, benzoic acid, methylhydantoin, and mevalonolactone (Schnurer and Magnusson, 2005). In addition, LAB can be used to reduce the contamination of grapes by mycotoxin. It has been reported that lactic acid bacteria (LAB) are used as a potential agent to minimize the contamination of mycotoxin and its toxicity (Dalié et al., 2010). Moreover, certain LAB can have probiotic potential such as antimicrobial effect on common pathogens and ability to adhere and colonize the intestinal tract. The use of lactic acid bacteria as biopreservator agent and having probioiotic potential demonstrates that they can be promising biological agents for food safety. The aim of the present study was to investigate the antifungal activity of lactic acid bacteria autochthones bacteria in grape against mycotoxinogen molds, and to determine their potential probiotic effect, the property to detoxify ochratoxin A was also studied using performed bacteria.

Material and methods

Isolation and screening of lactic acid bacteria with antifungal activities

Lactic acid bacteria strain was isolated from two varieties of bunch of grapes (Cardinal and Red Globe) provided by local market according to conventional microbial analysis. After pressing and filtration, samples were diluted in sterile saline solution (9 g l^{-1} NaCl) and then plated on de Man, Rogosa and Sharpe (MRS) agar at 37 °C during 48 h (Biokar Diagnostics).

Molds target strains

OTA-producing strains of *Aspergillus niger aggrégats* and *Aspergillus carbonarius* were obtained from Research Unit of Human Nutrition and Metabolic Disorder, USCR Mass Spectrometry, Faculty of Medicine of Monastir.

Screening assays for antifungal activities of LAB against *Aspergillus niger aggrégats* and *Aspergillus carbonarius*

Overlay technique Two mycotoxinogen fungal strains *Aspergillus niger aggrégats* and *Aspergillus carbonarius* were chosen to test antifungal activities of lactic acid bacteria. The LAB strains were grown in MRS (Biokar, diagnostics) broth at 37 °C. *Aspergillus niger aggrégats* and *Aspergillus carbonarius* were cultivated on malt extract agar (ME) (Merck, Darmstadt, Germany) at 25 °C. Spore inocula were prepared by suspending the conidia in 0.9%NaCl solution with 0.2% Tween 80, and spore concentrations were determined by counting the spore on a cell of Malassez under an optical microscope. According to Schwenninger et al. (2005) and Schillinger and Villarreal (2010), lactic acid bacteria strains were set up 16 h in MRS broth before the assays in order to reach the lag phase of growth (OD600 = 0.4–0.5) then spot inoculated onto MRS agar plates and allowed to grow at 30 °C for up to 3 days. The plates then were overlaid with malt extract (ME) agar (0.7%, 9 ml) containing about 10^4 per ml spores of target indicator molds. After incubation for up to5 days at 25 °C, the plates were examined for the formation of inhibition zones around the bacterial

colonies. The degree of inhibition was calculated as the diameter of the zone of inhibition. The zones were scored against the following arbitrary scale: no inhibition observed, 1–5 mm zones of inhibition, +; 6–10 mm zone of inhibiton, ++; 11–15 mm zone of inhibition, +++; ≥ 16 mm zone of inhibition, ++++ (Rouse et al. 2008).

Determination of antifungal activity of culture supernatants

The LAB strains were grown in MRS broth at 37 °C. After 16 h of incubation, cells were centrifuged at 4000 rpm for 15 min and the cell-free culture supernatants were filter sterilized 0.22 mm (Millipore). PDA agar plates containing 10^4 spores per milliliter of each indicator molds were prepared. Wells with a diameter of 5 mm were cut in the agar using a sterile cork borer. Then, 100 μl of culture supernatants was filled in the wells followed by incubation at 25 °C for 48 h. The plates were examined for the formation of inhibition zones around the wells.

Identification of lactic acid bacteria strains with antifungal activities

All isolates showing antifungal activities were identified by partial16S rRNA sequencing. Total DNA was extracted according to CTAB/NaCl method described by Wilson (1987) and modified by using lysozyme (1 mg/ml) for cell lysis. 16S rDNA gene PCR amplification are released using the primers 16SF (AGAGTTTGATCCTGGCTCAG) and 16SR (CTACGGCTACCTTGTTACGA). PCR amplicons were sequenced at the Biogenouest sequencing platform in LMBA (campus unversitaire El Manar) and sequences were aligned on the NCBI website (http://www.ncbi.nlm.nih.gov/ BLAST) for species assignment.

Screening of probiotic properties from selected lactic acid bacteria

Antibacterial activities

For the detection of antibacterial activities of selected lactic acid bacteria strains, the agar well diffusion assay test was used (Tagg and McGiven, 1971, Tagg et al., 1976). Molten agar (7.5 g/l) containing each indicator microorganism, namely *Klebsiella pneumoniae* ATCC 10031, *Staphylococcus aureus* ATCC 6138, *Bacillus cereus* ATCC 11778, *Pseudomonas aeruginosa* ATCC 9027, and *Escherichia coli* ATCC 10536, was spread over the surface of agar plates containing soy tripto-casein agar (TSA) medium and left to solidify. After solidification, a hole was made in the center of the plate and 80 μl of each supernatant (16–18 h) of the different lactobacilli strains was placed in the hole and allowed to diffuse at 4 °C for 2 h. After an overnight incubation at 37 °C, the plates were examined for halos around the hole.

Hydrophobicity test

The degree of hydrophobicity of the isolates was determined by employing the method described by Thapa et al. (2004), based on adhesion of cells to organic solvents. Solvent used were xylene (an apolar solvent), chloroform (a monopolar and acidic solvent), and ethyl acetate (a monopolar and basic solvent). The hydrophobicity of strain adhering to solvent was calculated as % Hydrophobicity = (1 − A1/A0) × 100.

Acid tolerance

According to Guo et al. (2009), selected LAB strains were grown in MRS broth at 37 °C for 18 h and centrifuged at 2500×*g* at 4 °C for 10 min. After two washing with PBS, cells were inoculated to PBS buffer solution (0.8% de NaCl; 0.02% of KH2PO4 and 0.115% of Na_2HPO4 (*p*/*v*) adjusted at different pH (pH 2.5, 3.0, 4.0) and incubated at 37 °C for 0, 1, 2, 3, and 4 h reflecting the time spent by food in the stomach. Viable colony counts of tested strains were enumerated on MRS agar after incubation anaerobically at 37 °C for 48 h. Survival rate was calculated according to the following eq. (Guo et al. 2009):

$$\text{Survival rate } \% = \log \text{ cfu N1} / \log \text{ cfuN0 x100}$$

N1 the total viable count of probiotic strains after treatment by PBS (pH 2.5, 3.0, 4.0).
N0 the total viable count of probiotic strains before treatment.

Determination of bile tolerance of cultures

The effect of bile salts on the growth rate of tested LAB strains was studied using the methods described by Walker and Gilliland (1993). The difference in time (h) between the culture media (with and without bile salts) was considered as the delay of growth (DG) (Usman, 1999).

Ochratoxin A removal by selected lactic acid bacteria

The potential of LAB strain *Pediococcus pentosaceus* (RG7B) for OTA reduction was evaluated in both MRS broth and sodium phosphate buffer (PBS) (Fuchs et al. 2008). The pH of both media was adjusted to 5.0 adding HCl (5 N) and was measured after autoclaving (121 °C; 15 min). *Pediococcus pentosaceus* (RG7B) was grown on MRS broth then incubated for 24 h at 37 °C. For

reduction experiments, a stock solution of OTA in methanol (10 μg/mL, Biopure corporation, USA) was used to supplement media to a final concentration of 50 ng/mL.

Bacterial cell absorbance was measured at 600 nm and cell pellets provided from an appropriate volume of *Pediococcus pentosaceus* suspension were used as inoculums of 5 ml of MRS broth and PBS to obtain a final bacterial concentration of 10^7 cells/ml. Each experiment was performed in triplicate.

The incubation was performed at 30 °C during 48 h on a horizontal shaker table (IKA, Staufen, Germany) with soft agitation (400 rpm). Positive controls, i.e., MRS and PBS, supplemented with OTA, but without bacteria, were incubated at the same conditions. After incubation, 2 ml of each tube was centrifuged at 10,000 rpm, 4 °C, 10 min, and the supernatants were transferred to Eppendorf vials and stored at 4 °C until HPLC analysis. Determination of OTA content in media before incubation, in control, and in cultures with LAB was carried out. The decrease of OTA by LAB in percentage was determined by subtracting the sample value from the value of the control sample (Fuchs et al., 2008).

Detection of OTA by HPLC-FLD

OTA analysis in the medium was realized as described by Fuchs et al. (2008) with some modification. A volume of 500 μl of the supernatants was diluted with 500 μl of methanol, homogenized with Ultra Turax then filtered (Millex^R SLHV 013NK, Millipore, Bedford, Massachusetts, U.S.A.) and directly used for OTA analysis. OTA detection and quantification were made by high-performance liquid chromatography (HPLC) equipped with a C_{18} column (Waters Spherisorb 5 μm, ODS2, 4.6 × 250 mm). The OTA detection was performed with fluorescence detection (Waters 474, Milford, Massachusetts, USA) at λ_{exc}330 nm and λ_{em}460 nm. The mobile phase was constituted with acetonitrile-water-acetic acid (57:41:2) with a flow rate of 1.0 ml/min. The injection volume was 25 μl and the retention time was 7 min. The detection limit was 5 ng OTA/ml based on a signal-to-noise ratio of 3:1 and the recovery rate was of 99%. The OTA quantification was realized with Empower (Waters, Milford) software, based on a calibration curve obtained by analyzing the peak area of OTA standard solutions diluted in methanol of six concentrations in the range between 5 and 1000 ng/ml.

Statistical analysis

Ochratoxin A concentrations detected in each treatment were evaluated by analysis of variance (ANOVA) using the Statistica software (version 5.0, StatSoft, Inc., Tulsa, OK, USA). Duncan's multiple range tests were used to assess the differences among the factor levels studied. Diameters of inhibition zone values in graphs are presented in the form of mean ± standard deviation (SD).

Results

Isolation of lactic acid bacteria strain

A total of 18 Gram-positive and catalase negative rods and cocobacilli were isolated from 22 samples of two varieties of table grape sold in the Tunisian market. All isolates were preliminary identified as LAB using the criteria of being Gram positive and catalase negative and by simple biochemical characterization (growth at 15 and 45 °C, CO2 production from glucose, and NH3 from arginine; Sharpe, 1979). Twelve strains were isolated from Red glob variety and 6 strains from Cardinal variety.

Screening assay for antifungal activities of lactic acid bacteria isolates against *Aspergillus niger aggrégats* and *Aspergillus carbonarius*

Overlay technique (culture of 3 days)

Among 18 lactic acid bacteria strains tested, 12 isolates exhibited inhibitory activity against *Aspergillus niger aggrégats*; the most performing isolate was RG7B showing the highest zone of inhibition (32 mm) (Table 1) (Fig. 1). Seven isolates exhibited a good inhibitory effect with zone of inhibition focused between 20 and 30 mm of diameter, whereas sex isolates had no inhibitory effect. Two isolates RG7B and C11C exhibited high antifungal activities against *Aspergillus carbonarius* showing inhibition zone over 25 mm.

Determination of antifungal activity of cell-free culture supernatants

None of the tested cell-free supernatants showed antifungal activities against *Aspergillus niger aggrégats* and *Aspergillus carbonarius*.

Strain identification

Selected isolates with antifungal properties were identified by complete 16S r-RNA sequence analysis after their phenotypic characterization (Table 1).

Screening of probiotic properties from lactic acid bacteria strains with antifungal activities

Six lactic acid bacteria were selected to test their potential probiotic activities and antimicrobial properties against a

Table 1 Antifungal activity of isolated LAB against *Aspergillus niger aggrégats* and *Aspergillus carbonarius* and molecular identification

Lab isolates	Indicator fungus		Molecular identification		
	Aspergillus niger	*Aspergillus carbonarius*	Genera/species	% of the similarity	Isolate accession numbers)
RG2A	+	+	*ND*		
RG3A	–	–	*ND*		
RG3B	+	–	*ND*		
RG4A	++	++	*Pediococcus. pentosaceus*	99%	MH555084
RG6A	+	+	*ND*		
RG6B	–	+	*ND*		
RG6C	++	–	*ND*		
RG7A	++	–	*ND*		
RG7B	+++	+++	*Pediococcus pentosaceus*	99%	MH555085
RG8A	–	++	*Lactobacillus plantarum*	98%	MH555088
RG8B	–	++	*ND*		
RG8C	–	++	*Pediococcus pentosaceus*	99%	MH555086
C7A	++	++	*Pediococcus pentosaceus*	99%	MH555083
C9C	+	+	*ND*		
C11A	++	+	*ND*		
C11B	–	–	*ND*		
C11C	++	+++	*Pediococcus pentosaceus*	99%	MH555087
C11D	++	–	*ND*		

panel of pathogenic bacteria, their ability to resist to acid and bile salt, and the degree of their hydrophobicity.

Antimicrobial activities

Among the pathogenic bacteria tested, only *Bacillus cereus* ATCC 11778 was inhibited by selected lactic acid bacteria strains; we focus moderate zone of inhibition ranged between 7.5 and 12 mm. No inhibition zone was detected against *Klebsiella pneumoniae* ATCC 10031, *Staphylococcus aureus* ATCC 6138, *Pseudomonas aeruginosa* ATCC 9027, and *Escherichia coli* ATCC 10536. In addition, the antimicrobial activity noted against *Bacillus cereus* ATCC 11778 was significantly decreased after neutralization and no inhibition was observed when the pH was adjusted to 7.0. Antimicrobial activities of LAB against *Bacillus cereus* ATCC 11778 are shown in Fig. 2.

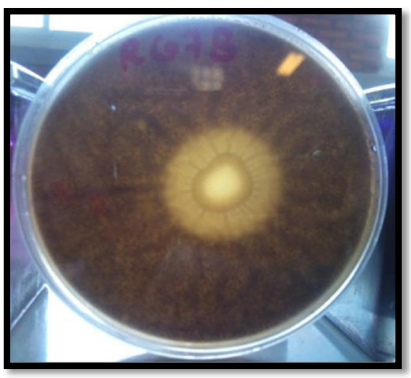

Fig. 1 Inhibition zone of RG7B against *Aspergillus niger aggrégats*

Hydrophobicity test

Selected isolates showed variable % hydrophobicity in the xylene, chloroform, and ethyl acetate (Fig. 3). However, all the tested strains presented higher affinities for chloroform similar to % hydrophobicity observed with control commercial probiotic bacteria *Lactobacillus plantarum* 299v. Moreover, RG8A showed the highest % hydrophobicity to chloroform (95,9%). In the contrast, all strains exhibited weak % hydrophobicity to the second solvent (ethyl acetate), ranged between 25.77 and 55.89%. In xylene, the majority of selected strains showed levels of % hydrophobicity higher than 45% (RG8A, RG8C, C7A and C11C, RG7B). No considerable differences in the levels of % hydrophobicity between these tested strains and commercial ones were observed which presented a value of 50.41% (Fig. 3).

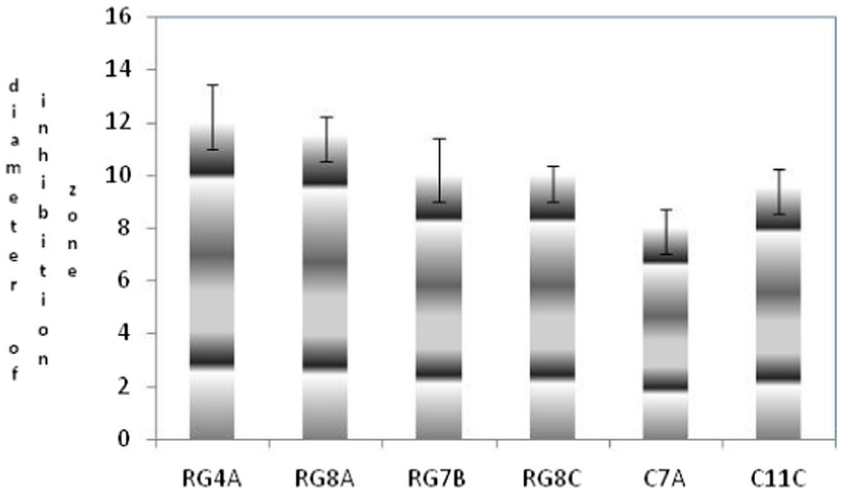

Fig. 2 Inhibition zone showed against *Bacillus cereus* ATCC 11778

Bile salt tolerance

All the tested strains were able to grow in the presence of bile salt, but the delay in the growth rate between cultures in MRS with and without bile salt are variable. The most tolerant strains were RG8A and RG4A with the lowest values of the delay growth 7.33 and 9.66 min, respectively (Table 2). RG7B and Lb 299v showed the highest delay of growth (27 and 30 min), respectively.

Acid tolerance

The survival rate of selected isolates in PBS buffer solution with low pH (2.5, 3, and 4) are presented in Fig. 4. We observed that the degree of viability of all the strains is better in pH 4 and 3 compared to pH 2.5 despite the variation in the degree of viability among the strains. The most acid-tolerant strains were RG8A and RG7B showing a survival rate in PBS at pH 2.5 after 4 h over 50%. The most acid-sensitive strain was RG4A and C11C at pH 3.

Reduction of Ochratoxin A by selected lactic acid bacteria

Results of the ability of the lactic acid bacteria *Pediococcus pentosaceus* RG7B to reduce Ochratoxin A concentration in liquid media are shown in Fig. 5. Statistical analyses showed that all single factors (media, bacteria, incubation) and their interaction significantly affect the OTA concentration ($P < 0.001$). It is worth noting that an OTA decrease was observed in control media not supplemented with bacteria with respect to initial OTA concentration. This reduction was about 27 and 16% in MRS and phosphate-buffered saline (PBS) media, respectively. Moreover, *Pediococcus pentosaceus* RG7B bacterial strain showed a significant high ability for OTA removal after 48-h incubation in both MRS and PBS media. However, the OTA removal was significantly higher in MRS than in

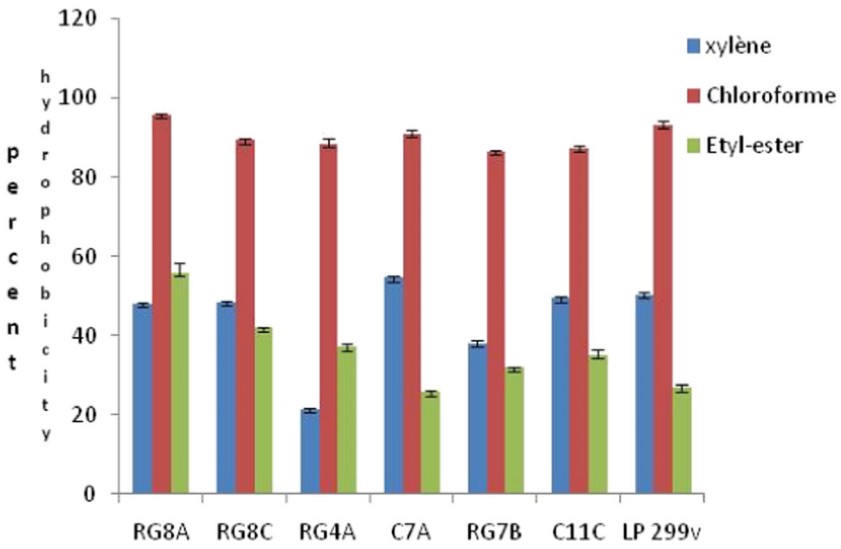

Fig. 3 Percent hydrophobicity of the isolates with various solvents

Table 2 Effect of bile salts on the growth rate of selected strains

LAB Isolates	Delay in the growth rate DG (min)
RG8C	20 ± 0,8
RG8A	7,33 ± 0,6
RG4A	9 ± 2,02
RG7B	27 ± 1,2
C11C	16 ± 0
Lb 299v	30 ± 0
C7A	15 ± 0,6

The measurements expressed in min are the mean of three replicates ± SD

PBS media with, respectively, a decrease percentage about 84 and 25% with respect to the control.

Discussion

Pioneering work on grape microbial ecology describes the main yeast species isolated from grapes and their environs (as reviewed by Fleet et al., 2002), whereas bacteria have been less studied (Barata et al., 2012). Our results showed that from two varieties of bunch of grapes (Cardinal and Red Globe), only 18 LAB strains were isolated. As reported, lactic acid bacteria are minor partners of grape microbiota (Barata et al., 2012). Bacterial populations are usually several orders of magnitude lower than those of yeasts in sound grapes. Lactic acid bacteria have counts lower than 10^2 CFU/g (Francesca et al., 2011).

Our previous isolation LAB from grape consortium represents a first step to investigate the capacities of indigenous LAB to inhibit its fungi contaminant. As known, lactic acid bacteria can be used as agent of biopreservation of food. They are considered to be active against a large range of food contaminant microorganism (e.g., molds) by the production of antimicrobial substances like organic acids and bacteriocins (Belkacem-Hanfi et al., 2014).

In this study, we tested all LAB strains previously isolated from grape against the most contaminant molds such as *Aspergillus niger aggrégats* and *Aspergillus carbonarius*. The genus Aspergillus is a component of the epiphytic flora of grapes and may be present on grape berries (Roussaut et al., 2014). The genus *Aspergillus* represents the most mold contaminant in grape from Mediterranean. *Aspergillus carbonarius* and *Aspergillus niger* are the main OTA producers commonly detected in grapes in the Mediterranean area (Covarelly et al. 2015).

Selected LAB strains with antifungal activities were identified as *Pediococcus pentosaceus* and *Lactobacillus plantarum* group (RG8A) on the basis of 16SRNA gene sequencing. Several studies showed the predominance of *Pediococcus pentosaceus* in cereal products or in fermented vegetables (Jonganurakkun et al., 2008; Huang et al., 2009). In contrast, there are a few studies which describe the presence of this strain in grape products. Renouf et al., 2005, Renouf and Lonvaud-Funel, 2007) reported that the *Pediococcus* genera, particularly *Pediococcus parvulus*, *P. damnosus*, and *P. acidilactici*, was isolated from grape. Crowley et al. (2013) reported that *P. pentosaceous* was shown to have protective properties against *P. expansum* spoilage when applied in pear, plum, and grape models.

In the limited available literature, *Lactobacillus plantarum* was found to be the most abundant species isolated from grape

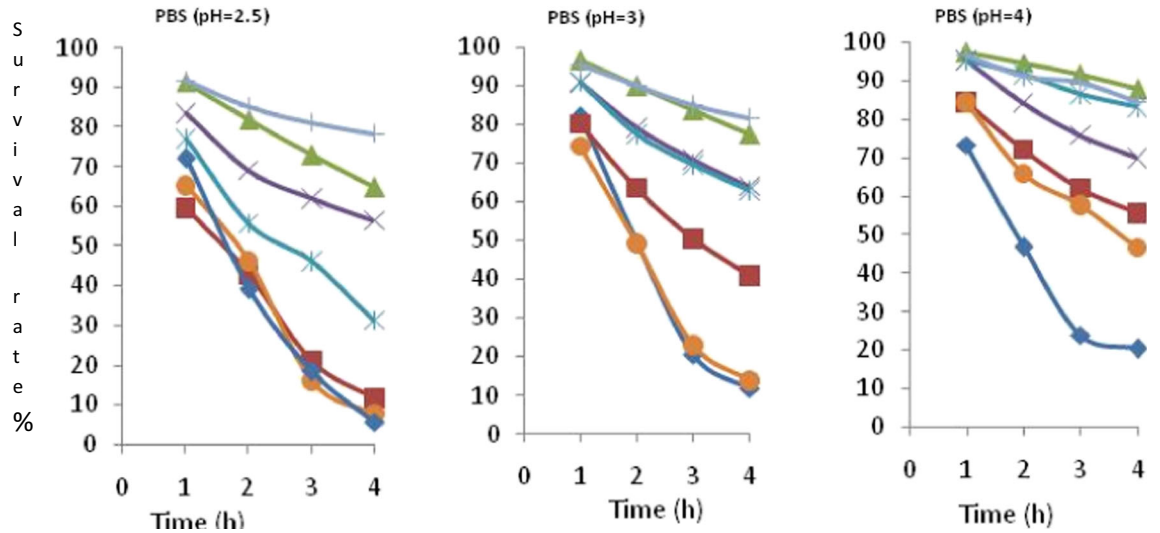

Fig. 4. Tolerance of selected strains *Pediococcus pentosaceus* C11C (blue diamond), *Pediococcus pentosaceus* RG7B (cross mark), *Lactobacillus plantarum* RG8A (green triangle), *Pediococcus pentosaceus* C7A (brown square), *Pediococcus pentosaceus* RG8C (light blue asterisk) *Pediococcus pentosaceus* RG4A (orange circle), *Lactobacillus plantarum* Lb 299v (blue batton) to PBS adjusted as acid pH. [a,b,c]Values with different superscript are significantly different ($P < 0.05$).

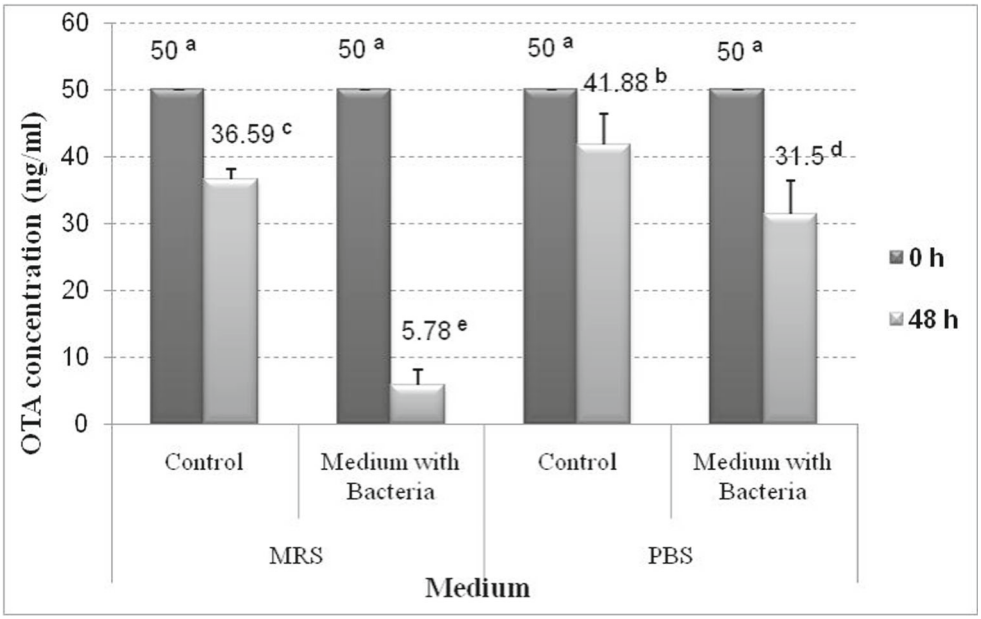

Fig. 5 Ochratoxin A reduction by *Pediococcus pentosaceus* (RG7B) after 48 h of incubation (30 °C) in MRS and PBS media. Control (medium without addition of bacterial suspension) and medium with bacteria (medium supplemented with bacteria at 10^7CFU/ml)

[a,b,c] Values with different superscript are significantly different ($P<0.05$)

(De Pina and Hogg, 1999; Silva and Malcata, 2000). However, this specie is widely used in fermentations of vegetables (Leal-Sánchez et al., 2003; Argyri et al., 2013). As reported by Maragkoudakis et al. (2013), the dominance of *L. plantarum* in non-acidified grape marc during storage could be due to various reasons such as their natural ability to withstand mild-to-low pH values and biofilm formation ability. Lavermicocca et al. (2000), Strom et al. (2002), Yang and Chang (2010) reported that *L. plantarum* inhibits efficiently many species of *Aspergillus.* In addition, *L. plantarum* was shown to be active against spoilage filamentous fungi typically found in baked goods such as *Penicillium* spp. and *Fusarium* spp. (Dal Bello et al. 2007; Gerez et al. 2010; Russo et al. (2016). On the other hand, cell-free supernatant of LAB tested did not show any antifungal activities against *Aspergillus niger aggrégats* and *Aspergillus carbonarius*. The inhibition effect was only detected with overlay assay, indicating that inhibitory activity is due to the presence of bacterial cells and not to metabolic products of the LAB. As reported previously (Schnürer, Magnusson, 2005), LAB can produce a range of antifungal substances including organic acids, proteinaceous compounds, and low-molecular-weight substances, e.g., phenyllactic acid, reuterin, cyclic peptides, 3-hydroxylated fatty acids, benzoic acid, methylhydantoin, and mevalonolactone. It is the first report showing that the inhibition of molds such as *Aspergillus niger aggrégats* and *Aspergillus carbonarius* is probably due to the presence of bacterial cells and not to metabolic substances produced by LAB strains.

As shown by Crowley et al. (2013), a large number of LAB isolates were subsequently identified to produce a broad spectrum of activity against *Penicillium expansum*, *Penicillium digitatum*, *Penicillium notatum*, *Penicillium roqueforti*, *Rhizopus stolonifer*, *Fusarium culmorum*, *Aspergillus fumigatus*, and *Rhodotorula mucilaginosa* and the antifungal compound(s) was shown to be neither proteinaceous nor volatile in nature. Thus, it will be interesting to investigate the mechanisms induced by the inhibition of mold by LAB and then to characterize antifungal responsible compounds. Similar results confirmed that antifungal activities of LAB may be explained by a variety of hypotheses; Schnürer and Magnusson (2005) reported that the inhibition of mold growth on an agar plate is the result of a complex interaction of numerous compounds and metabolites contributing to the overall antifungal activity. Many of them are difficult to detect. Further studies on the isolation and identification of bioactive compounds responsible for the inhibition of mold growth are needed. On the other hand, numerous investigations indicate that LAB have beneficial health effects in humans (Ouwehand et al. 2002; Saxelin et al. 2005). According to the current adopted definition by FAO/WHO (2001), "probiotics are live microorganisms which when administered in adequate amounts confer a health benefit on the host". Given this, our selected strains with antifungal properties were screened to detect which one can present a probiotic potential. Criteria for selection of a probiotic vary but usually includes the ability to adhere to mucus and epithelial cells (Gorbach, 2002) and survival at low pH (1.0 to 3.0) and bile salts of approximately 0.3% (Mainville et al. 2005). To provide health benefits, a potential probiotic is required to have the ability to overcome physical and chemical barriers such as gastric, bile, and pancreatic secretions; attach

to epithelial cells; and colonize the human intestine (Del Piano et al. 2006). As regards the tolerance of gastric barrier, two strains RG8A and RG7B were able to resist low pH environment without a high loss of viable cells; as known, food normally remains in the stomach for 2–4 h (Huang &Adams, 2004), but in our case, RG7B and RG8A were highly tolerant during 4 h of contact as compared with commercial probiotic strain 299v. Our results showed that all tested strains were fully tolerant to 0.3% bile salts given that they showed a delay growth lower than 40 min according to the classification of Gilliland et al. (1984) and Chateau et al. (1994). To test bacterial properties of adhesion, it is essential to know their physiochemical surface properties including their Lewis acid base or electron acceptor/electron donor character. Therefore, quantitative technique, MATS (microbial adhesion to solvents), which was inspired by the MATH (microbial adhesion to hydrocarbons) method, was used according to Rosenberg et al. (1980). Cell surface hydrophobicity is explained by bacterial adhesion to xylene. As reported by Rijnaarts et al. (1993), the hydrophobicity of the cell increases the level of adhesion also increases. So, C7A, RG7B, RG8C, and RG8A strains showed good values of hydrophobicity as compared with hydrophobicity of probiotic strain 299v which reached 50.41%. In chloroform, all tested strains exhibited good adhesion properties, more than 85%, demonstrating that all strains are able to adhere. Antibacterial effect was also studied showing that only *Bacillus cereus* was inhibited by tested LAB, and in the second assay with neutralized supernatant, no inhibition is determined. It can be concluded that the inhibition is particularly due to production of lactic acid. Our findings were also in agreement with previous studies proposing that growth-inhibiting activity has generally been attributed to the fact that lactic acid bacteria lower pH and/or produce organic acids (Rossland et al. 2003). On the other hand, selected LAB strain *Pediococcus pentosaceus* (RG7B) with the highest antifungal activity and having promising probiotic potential was chosen to test its ability to detoxify mycotoxin such as OTA. Our results showed that the best removal capacity was shown after 48 h of incubation in MRS medium. This finding demonstrates that *Pediococcus pentosaceus* (RG7B) can remove the ochratoxin A present in the liquid media but how this phenomenon can be explicated is still unknown. In fact, the highest reduction of OTA concentration is shown in experiment realized in MRS after bacterial growth and not in PBS when cell pellet was directly in contact with OTA. It can be explained that metabolism of LAB strain was the mechanism by which OTA was removed from the media. Our findings were in contrast with results of Del Prete et al. (2007) and Fuchs et al. (2008) where they reported that binding and not metabolism was responsible of ochratoxin removal. In addition, Piotrowska and Zakowska (2005) and Niderkorn et al. (2009) reported that binding of mycotoxins to LAB peptidoglycan wall and/or surface proteins represent the mechanism of detoxification by LAB. As mentioned by Fuchs et al. (2008), OTA and PAT are removed by far more efficiently by viable bacteria which can be taken as an indication that processes other than binding to the cell walls, for example, metabolic conversion by the release of specific enzymes, are involved. Otherwise, in our experiment, we noticed that OTA amounts decreased after 48 h of incubation in control media not supplemented with bacteria with respect to initial OTA concentration (27 and 16% in MRS and PBS media, respectively). This decrease is probably due to the ability of some compounds of the medium to bind OTA which will be removed during filtration (0.45 μm).

Supplemental studies are necessary to further understand the mechanisms through which ochratoxin was removed from media. The possibility of using LAB with GRAS (generally regarded as safe) status and probiotic potential as a biotechnological solution to fungal spoilage and mycotoxin formation is a promising option for both the food industry and the agricultural sector. Moreover, interesting strains could be employed as a novel LAB starter with probiotic properties in grape beverage.

Conclusions

Screening of LAB with antifungal activities from grape branch against its most contaminant molds *Aspergillus niger aggrégats* and *Aspergillus carbonarius* showed that the performing strain was *Pediococcus pentosaceus* RG7B. This strain had not only a good capacity to survive under simulated digestive tract such as gastric and intestinal juice but also high values of hydrophobicity, so it can be considered as potential probiotic candidate. In this study, we also demonstrate that *Pediococcus pentosaceus* RG7B has a good ability to remove ochratoxin A. Given this, development of probiotic beverage from grape juice using selected lactic acid bacteria and also having a property of detoxifying ochratoxin A may be a good and healthy alternative functional food containing probiotics.

Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

References

Argyri AA, Zoumpopoulou G, Karatzas KAG, Tsakalidou E, Nychas GJE, Panagou E, Tassou CC (2013) Selection of potential probiotic lactic acid bacteria from fermented olives by in vitro tests. Food Microbiol 33:282–291

Barata A, Malfeito-Ferreira M, Loureiro V (2012) The microbial ecology of wine grape berries. Int J Food Microbiol 153(3):243–259

Battilani P, Pietri A, Bertuzzi T, Languasco L, Giorni P, Kozakiewicz Z (2003) Occurrence of ochratoxin A–producing fungi in grapes grown in Italy. J Food Prot, 4, 0 535-709, 633-636(4)

Battilani P, Giorni P, Bertuzzi T, Formenti S, Piertri A (2006) Black aspergilli and ochratoxin A in grapes in Italy. Int J Food Microbiol 111:S53–S60

Belkacem-Hanfi N, Fhoula I, Semmar N, Guesmi A, Perraud-Gaime I, OuzariHadda I, Boudabous A, Roussos S (2014) Lactic acid bacteria against post-harvest moulds and ochratoxin A isolated from stored wheat. Biol Control 76:52–59

Bellí N, Ramos AJ, Sanchis V, Marin S (2004a) Incubation time and water activity effects on ochratoxin A production by *Aspergillus* section *Nigri* strains isolated from grapes. Lett Appl Microbiol 38: 72–77

Bellí N, Marín S, Duaigües A, Ramos AJ, Sanchis V (2004b) Ochratoxin A in wines, musts and grape juices from Spain. J Sci Food Agric 84(6):591–594

Burdaspalp A, Legarda M (1999) Ochratoxin A in wines and grape products originated from Spain and other european countries. Alimentaria 299:107–113

Cabañes FJ, Accensi F, Bragulat MR, Abarca ML, Castellá G, Minguez S, Pons A (2002) What is the source of ochratoxin A in wine ? Int J Food Microbiol 79(3):213–215

Chateau N, Deschamps AM, Hadj Sassi A (1994) Heterogeneity of bile salts resistance in the Lactobacillus isolates of a probiotic consortium. Lett Appl Microbiol 18(1):42–44

Covarelli L, Tosi L, Beccari G (2015) Chapter 50 - Risks Related to the Presence of Fungal Species and Mycotoxins in Grapes, Wines and Other Derived Products in the Mediterranean Area. The mediterranean diet 563–575.

Creppy EE (1998) Human ochratoxicosis and nephropathy in Egypt. Hum ExpToxicol 17:124–129

Crowley S, Mahony J, van Sinderen D (2013) Broad-spectrum antifungal-producing lactic acid bacteria and their application in fruit models. Folia Microbiology 58(4):291–299

Dal Bello F, Clarke CI, Ryan LAM, Ulmer H, Schober TJ, Ström K, Sjögren J, vanSinderen D, Schnürer J, Arendt EK (2007) Improvement of the quality and shelf life of wheat bread by fermentation with the antifungal strain *Lactobacillus plantarum* FST 1.7. J. Cereal Science 45:309–318

Dalié DKD, Deschamps AM, Richard-Forget F (2010) Lactic acid bacteria—potential for control of mould growth and mycotoxins. Food Control 21:370–380

De Pina CG, Hogg TA (1999) Microbial and chemical changes during the spontaneous ensilage of grape marc. J Appl Microbiol 86:777–784

Del Piano M, Morelli L, Strozzi GP, Allesina S, Barba M, Deidda F, Lorenzini P, Ballar´e M, Montino F, Orsello M, Sartori M, Garello E, Carmagnola S, Pagliarulo M, Capurso L (2006) Probiotics: from research to consumer. Dig Liver Dis 38:248–255

FAO/WHO (2001) Report of a joint FAO/WHO expert consultation on evaluation of health and nutritional properties of probiotics in food including powder milk with live lactic acid bacteria. Cordoba, Argentina

Filali A, Ouammi L, Betbeder AM, Baudrimont I, Soulaymani R, Benayada A, Creppy EE (2001) Ochratoxin A in beverages from Morocco: a preliminary survey. Food Addit Contam 18(6):565–568

Fleet, G. H., Prakitchaiwattana, C., Beh, A.L., Heard, G. (2002). The yeast ecology of wine grapes. Biodiversity and Biotechnology of Wine Yeasts Research Signpost, 1–17

Francesca N, Settanni L, Sannino C, Aponte M, Moschetti G (2011) Ecology and technological capability of lactic acid bacteria isolated during Grillo grape vinification in the Marsala production area. Ann Microbiol 61:79–84

Fuchs S, Sontag G, Stidl R, Ehrlich V, Kundi M, Knasmuller S (2008) Detoxification of patulin and ochratoxin A, two abundant mycotoxins, by lactic acid bacteria. Food Chem Toxicol 46:1398–1407

Gerez CL, Torino MI, Obregozo MD, de Valdez G (2010) A ready-to use antifungal starter culture improves the shelf life of packaged bread. J Food Prot 73:758–762

Gilliland SE, Staley TE, Bush LJ (1984) Importance of bile tolerance of Lactobacillus acidophilus used as dietary adjunct. J Dairy Sci 67: 3045–3051

Gómez C, Bragulat MR, Abarca ML, Mínguez S, Cabañes FJ (2006) Ochratoxin A-producing fungi from grapes intended for liqueur wine production. Food Microbiol 23(6):541–545

Gorbach SL (2002) Probiotics in the third millennium. Dig Liver Dis 34(Suppl.2):52–57

Guo Z, Wang J, Yan L, Chen W, Liu X-m, Zhang H-p (2009) In vitro comparison of probiotic properties of Lactobacillus casei Zhang, a potential new probiotic, with selected probiotic strains. LWT Food Sci Technol 42:1640–1646

Huang Y, Adams MC (2004) In vitro assessment of the upper gastrointestinal tolerance of potential probiotic dairy propionic bacteria. Int J Food Microbiol 91(3):253–260

Huang Y, Lu Y, Zhai ZH, Yang C, Hongtao T, Zheng L, Feng J, Liu H, Hao Y (2009) Characterization and application of an anti-*Listeria* bacteriocin produced by *Pediococcus pentosaceus* 05-10 isolated from Sichuan pickle, a traditionally fermented vegetable product from China. Food Control 20(11):1030–1035

IARC: International Agency for Research on Cancer (1993) Some naturally occurring substances: food items and constituents, heterocyclic aromatic amines and mycotoxins. Monogr Eval Carcinog Risks Hum 56:489–521

Jonganurakkun B, Wang Q, Xu SH, Tada Y, Minamida K, Yasokawa D, Sugi M, Hara H, Asano K (2008) Pediococcus pentosaceus NB-17 for probiotic use. J Biosci Bioeng 106(1):69–73

Krogh P (1992) Role of ochratoxin in disease causation. Food Chem Toxicol 3:213–224

del Prete V, Rodriguez H, Carrascosa AV, de las Rivas B, Garcia-Moruno E, Munoz R (2007) In vitro removal of ochratoxin A by wine lactic acid bacteria. J Food Prot 70:2155–2160

Lasram S, Oueslati S, Mliki A, Ghorbel A, Silar P, Chebil S (2012) Ochratoxin A and ochratoxigenic black Aspergillus species in Tunisian grapes cultivated in different geographic areas. Food Control 25:75–80

Lavermicocca P, Valerio F, Evidente A, Lazzaroni S, Corsetti A, Gobbetti M (2000) Purification and Characterization of Novel Antifungal Compounds from the Sourdough Lactobacillus plantarum Strain 21B. Appl Environ Microbiol 66:4084–4090

Leal-Sánchez MV, Ruiz-Barba JL, Sánchez AH, Rejano L, Jiménez-Díaz R, Garrido A (2003) Fermentation profile and optimization of green olive fermentation using lactobacillus plantarum LPCO10 as a starter culture. Food Microbiol 20:421–430

Leong S-L, Hocking AD, Pitt JI (2004) Occurrence of fruit rot fungi (*Aspergillus section Nigri*) on some drying varieties of irrigated grapes. Aust J Grape Wine Res 10(1):83–88

Mainville I, Arcand Y, Farnworth ER (2005) A dynamic model that simulates the human upper gastro-intestinal tract for the study of probiotics. Int J Food Microbiol 99:287–296

Maragkoudakis PA, Nardi T, Bovo B, D'Andrea M, Howell KS, Giacomini A, Corich V (2013) Biodiversity, dynamics and ecology of bacterial community during grape marc storage for the production of grappa. Int J Food Microbiol 162:143–151

Niderkorn V, Morgavi DP, Aboab B, Lemaire M, Boudra H (2009) Cell wall component and mycotoxin moieties involved in the binding of fumonisin B1 and B2 by lactic acid bacteria. J Appl Microbiol 106: 977–985

Ouwehand AC, Salminen S, Isolauri E (2002) Probiotics: an overview of beneficial effects. Antonie Van Leeuwenhoek 82:279–289

Peyer LC, Axel C, Lynch KM, Zannini E, Fritz J, Arendt EK (2016) Inhibition of Fusarium culmorum by carboxylic acids released from

lactic acid bacteria in a barley malt substrate. Food Control 69:227–236

Piotrowska M, Zakowska Z (2005) The elimination of ochratoxin A by lactic acid bacteria strains. Pol J Microbiol 54:279–286

Reis JA, Paula AT, Casarotti SN, Penna ALB (2012) Lactic acid bacteria antimicrobial compounds: characteristics and applications. Food Eng Rev 4(2):124–140

Renouf V, Lonvaud-Funel A (2007) Development of an enrichment medium to detect *Dekkera*/*Brettanomyces bruxellensis*, a spoilage wine yeast, on the surface of grape berries. Microbiol Res 162(2):154–167

Renouf V, Claisse O, Lonvaud-Funel A (2005) Understanding the microbial ecosystem on the grape berry surface through numeration and identification of yeast and bacteria. Aust J Grape Wine Res 11(3): 316–327

Rijnaarts HHM, Norde W, Bouwer EJ, Lyklema J, Zehnder AJB (1993) Bacterial adhesion under static and dynamic conditions. Appl Environ Microbiol 59:3255–3265

Rosenberg M, Gutnick D, Rosenberg E (1980) Adherence of bacteria to hydrocarbons: a simple method for measuring cell-surface hydrophobicity. FEMS Microbiol Lett 9:29–33

Rossland E, Borge GIA, Langsrud T, Sorhaug T (2003) Inhibition of *Bacillus cereus* by strains of *Lactobacillus* and *Lactococcus* in milk. Int J Food Microbiol 89:205–212

Rouse S, Canchaya C, van Sinderen D (2008) *Lactobacillus hordei*sp nov a bacteriocinogenic strain isolated from malted barley. Int J Syst Evol Microbiol 58:2013–2017

Rousseaux S, Filofteia DC, Radoï-Matei F, Alexandre H, Guilloux-Bénatier M (2014) Non-botrytis grape-rotting fungi responsible for earthy and moldy off-flavors and mycotoxins. Food Microbiol 38: 104–121

Russo, P., Arena, M, Fiocco, D., Capozzi, V, Drider, D., Spano, G. (2016). *Lactobacillus plantarum* with broad antifungal activity: a promising approach toi ncrease safety and shelf-life of cereal-based products International Journal of Food Microbiology in press

Saxelin M, Tynkkynen S, Mattila-Sandholm T, de Vos WM (2005) Probiotic and other functional microbes: from markets to mechanisms. Curr Opin Biotechnol 16(2):204–211

Schillinger U, Villarreal JV (2010) Inhibition of *Penicillium nordicum* in MRS medium by lactic acid bacteria isolated from foods. Food Control 21(2):107–111

Schnurer J, Magnusson J (2005) Antifungal lactic acid bacteria as biopreservatives. Trends Food Sci Technol 16:70–78

Schwenninger SM, von Ah U, Niederer B, Teuber M, Meile A (2005) Detection of antifungal properties in *Lactobacillus paracasei subsp. paracasei* SM20, SM29, and SM63 and molecular typing of the strains. J Food Prot 68(1):111–119

Serra R, Braga A, Venâncio A (2005) Mycotoxin-producing and other fungi isolated from grapes for wine production, with particular emphasis on ochratoxin A. Res Microbiol 156:515–521

Sharpe, M. E. (1979). Identification of the lactic acid bacteria. In F. A. Skinner & D. W.Lovelock (Eds.), identification methods for microbiologists (2nd ed.). London:Academic Press

Silva ML, Malcata FX (2000) Effect of time and temperature of fermentation on the microflora of grape marc. Bioprocess Biosyst Eng 23: 17–24

Strom K, Sjogren J, Broberg A, Schnurer J (2002) Lactobacillus plantarum MiLAB 393 Produces the Antifungal Cyclic Dipeptides Cyclo(L-Phe–L-Pro) and Cyclo(L-Phe–trans-4-OH-L-Pro) and 3-Phenyllactic Acid. Appl Environ Microbiol 68:4322–4327

Tagg JR, McGiven AR (1971) Assay system for bacteriocins. Appl Microbiol 21:943

Tagg JR, Dajani AS, Wannamaker LW (1976) Bacteriocins of gram-positive bacteria. Bacteriol Rev 40:722–756

Thapa N, PaL J, Tamang JP (2004) Microbial diversity in ngari, hentak and tungtap, fermented fish products of north-east India. World J Microbiol Biotechnol 20:599–607

Tjamos SE, Antoniou PP, Tjamos E (2006) *Aspergillus* spp., distribution, population composition and ochratoxin A production in wine producing vineyards in Greece. Int J Food Microbiol 111(1):S61–S66

Usman HA (1999) Bile tolerance, taurocholate deconjugation, and binding of cholesterol by lactobacillus gasseristrains. J Dairy Sci 82(2): 243e248

Walker DK, Gilliland SE (1993) Relationships among bile tolerance, bile salts deconjugation and assimilation by cholesterol by lactobacillus acidophilus. J Dairy Sci 76:956–961 webmanagercenter.com 18 Août 2015: Tunisie , Economie, raisins , Agriculture

Wilson K (1987) Preparation of genomic DNA from bacteria. In: Ausubel FM, Bent R, Kingston RE et al (eds) Current protocols in molecular biology. J. Wiley & Sons, New York, N.Y, pp 2.10–2.12

Yang EJ, Chang HC (2010) Purification of a new antifungal compound produced by *Lactobacillus plantarum* AF1 isolated from kimchi. Int J Food Microbiol 139(1–2):56–63