LWT - Food Science and Technology 81 (2017) 128–135

Contents lists available at ScienceDirect

LWT - Food Science and Technology

journal homepage: www.elsevier.com/locate/lwt

In vitro antifungal activity of bioactive peptides produced by *Lactobacillus plantarum* against *Aspergillus parasiticus* and *Penicillium expansum*

C. Luz [a], F. Saladino [a], F.B. Luciano [b], J. Mañes [a], G. Meca [a, *]

[a] Laboratory of Food Chemistry and Toxicology, Faculty of Pharmacy, University of Valencia, Av. Vicent Andrés Estellés s/n, 46100 Burjassot, Spain
[b] School of Life Sciences, Pontifícia Universidade Católica do Paraná, Curitiba, Paraná, Brazil

ARTICLE INFO

Article history:
Received 11 October 2016
Received in revised form
8 February 2017
Accepted 30 March 2017
Available online 31 March 2017

Keywords:
Antimicrobial peptides
Antifungal activity
Lactobacillus plantarum
Spoilage fungi

ABSTRACT

Food spoilage caused by mycotoxigenic moulds represents an important problem in food security. The antimicrobial peptides are compounds of natural origin constituted by a variable number (5–100) of amino acids held together through peptide bonds. In this work, the cell free supernatants (CFSs) containing peptides obtained from four strains of LAB were lyophilized, filtered and tested to determine the antifungal activity against *Aspergillus Parasiticus* and *Penicillium expansum*. CFS obtained by *Lactobacillus plantarum* showed the highest inhibition activity. CFS was fractionated by size exclusion chromatography and injected into the liquid chromatography coupled to diode array detector. One of the recollected fractions resulted interesting for the presence of three peaks that were purified by the technique of the LC-DAD using a semi preparative C18 column. Finally, the antifungal activity of the purified peptides was studied against *A. Parasiticus* and *P. expansum* in liquid medium. The MALDI-TOF/TOF mass spectrometry was used for the peptides identification. The three purified peptides presented an amino acidic sequence identified by a bioinformatics program of SGADTTFLTK, LVGKKVQTF, and GTLIGQDYK. The first peptide purified reduced 58% and 73% the growth of *P. expansum* and *A. parasiticus*, respectively, in liquid medium after 48 h incubation.

1. Introduction

Mould deterioration of food, feed and other agricultural commodities can be responsible for considerable economical losses. Thirty percent of crop yields are destroyed and more than 30% of perishable crops are damaged in developing countries by lowering their quality and quantity. Furthermore, moulds produce compounds potentially toxics to the consumers called mycotoxins which can cause illness and death (Pawlowska, Zannini, Coffey, & Arendt, 2012). High incidences of mould and mycotoxin contamination in food and feed are due to fungi ubiquitous nature, to their ability to colonize different substrates and to the lack of effective control measures (Hassan, Zhou, & Bullerman, 2015).

Currently, food industry depends on chemical preservatives to extend the shelf life and control the growth of spoilage fungi. The consumer's awareness about the health hazards associated with chemicals has recently increased, and they are demanding for processed foods that are free of preservatives. There are many natural alternative preservatives produced by certain microorganisms, however these microorganisms have to be non-toxic, easy to grow and require simple media for cultivation. Lactic acid bacteria (LABs) are a known potential source for generating a variety of secondary metabolites such as bacteriocines, organic acids and peptides (Cizeikiene, Juodeikiene, Paskevicius, & Bartkiene, 2013). In the past decade the interest for antifungal LABs has increased and different studies have showed that many LAB strains have the potential to combat the proliferation of fungi in various food and feed materials (Rouse, Harnett, Vaughan, & Van Sinderen, 2008; Dalie, Deschamps, & Richard-Forget, 2010; Mauch, Dal Bello, Coffey, & Arendt, 2010; Gerez, Torino, Rollan & De Valdez, 2009; Dal Bello et al., 2007). Consumers are demanding the replacement of artificial chemical preservatives by natural biopreservatives to reduce fungal contamination in foods (Brul & Coote, 1999; Crowley, Mahony, & Van Sinderen, 2013; Reis, Paula, Casarotti, & Penna, 2012; Schnurer & Magnusson, 2005). LABs, due to their long history of safe use in food and feed fermentations (Hugenholtz, 2013;

* Corresponding author.
E-mail address: giuseppe.meca@uv.es (G. Meca).

http://dx.doi.org/10.1016/j.lwt.2017.03.053

Stoyanova, Ustyugova, & Netrusov, 2012), have received both GRAS (Generally Recognised as Safe) and QPS (Qualified Presumption of Safety) status in the EU. Therefore, LABs have good potential for future as antifungal biocontrol agents.

Bacteriocins/antimicrobial peptides produced by LABs have been reported to be involved in the preservation of many processed and natural foods (Settanni & Corsetti, 2008; Bellei, Miguel, Mere Del Aguila, Silva, & Paschoalin, 2011; Udhayashree, Senbagam, Senthilkumar, Nithya, & Gurusamy, 2012). Their usage in the food industry has promoted the reduction of chemical preservatives and intense heat treatments, thus, resulting in foods which are more naturally-preserved and richer in nutritional properties (Coda et al., 2011; De Vuyst & Leroy, 2007; Parada, Caron, Medeiros, & Soccol, 2007). Pediocin PA-1/AcH is not allowed as a food additive but is only applied in form of protective cultures whereas nisin is the only commercially acceptable food-grade bacteriocin in use (Chen & Hoover, 2003; Díez et al., 2012; Settanni & Corsetti, 2008). In addition to these, other bacteriocins (lacticin 3147, enterocin AS-48 or variacin) also offer promising perspectives (Gálvez, Abriouel, López, & Omar, 2007). So it is meaningful and highly pertinent to screen for natural bioactive peptides to find novel ones for a specific application.

The antifungal effect by the culture supernatant of various LAB strains against few fungi including food-deteriogens has been worked out by various authors (Yang & Clausen, 2005; Sathe, Nawani, Dhakephalkar, & Kapadnis, 2007; Rouse, Canchaya, & Van Sinderen, 2008; Gerez, Torino, Rollan, & Valdez, 2009; Smaoui et al., 2009; Adebayo & Aderiye, 2010; Coda et al., 2011). Several compounds have been proposed as responsible for the antifungal activity of LAB, like organic acids, low molecular weight compounds, phenylacetic and fatty acids, cyclic dipeptides, proteinaceous compounds and other miscellaneous compounds e.g. lactones (Peyer et al., 2016). The fungistatic activity of pentocin TV35b against *Candida albicans* (Okkers, Dicks, Silvester, Joubert, & Odendaal, 1999) and of two LAB strains against *Fusarium* (isolated from cereals) has been reported (Laitila, Alakomi, Raaska, Mattila-Sandholm, & Haikara, 2002). Due to their inhibitory spectrum these peptides are consider to be powerful antimicrobials to target bacteria, fungi and parasites (Mason et al., 2009).

The aims of this study were a) to evaluate the antifungal activity of cell free supernatant containing peptides obtained from different LABs against *A. parasiticus* and *P. expansum*; b) to purify, isolate and identify the active compounds produced during the fermentation by the most potential LAB and c) to test each one of them against fungal growth of *A. parasiticus* and *P. expansum*.

2. Materials and methods

2.1. Chemicals

Acetonitrile was purchased from VWR (Leuven, Bélgica). Deionized water (<18 MΩ cm resistivity) was obtained from a Milli-Q water purification system (Millipore, Bedford, MA, USA). Chromatographic solvents and water were degassed for 20 min using a Branson 5200 (Branson Ultrasonic Corp., CT, USA) ultrasonic bath. Buffered peptone water, potato dextrose agar (PDA), phosphate buffer saline (PBS, pH 7.4), potato dextrose broth (PDB) and De Man Rogosa and Sharpe broth and agar (MRS broth and agar) were provided by Oxoid (Madrid, Spain).

2.2. Microorganisms and culture conditions

The strain of *A. parasiticus* CECT 2681 and *P. expansum* CECT 2268 were obtained from the Spanish Type Culture Collection (CECT, Valencia, Spain). These microorganisms were maintained in sterile glycerol at −80 °C. Then, they were recovered in PDB broth at 25 °C until they were inoculated in PDA Petri dishes.

The LABs used in this study were: *Lactobacillus rhamnosus* CECT 278T, *Lactobacillus johnsoni* CECT 289, *Lactobacillus plantarum* CECT 749, and *Lactobacillus delbrueckii bulgaricus* CECT 4005. These bacteria were also obtained from CECT Valencia, Spain. The LABs were preserved in sterile 18% glycerol and stored at −80 °C before use. They were recovered in MRS broth at 37 °C for 48 h under anaerobic conditions (Anaerocult A, Merk – Darmstadt, Germany) before experiments (Meroth, Walter, Hertel, Brandt, & Hammes, 2003). Bacteria concentration was measured by optical density at 600 nm using a spectrophotometer (Shimadzu UV mini 1240 – Columbia, EE.UU.) and adjusted to 10^6 LAB/mL to obtain the same initial conditions for all the bacteria. After the incubation period, the cells were removed by centrifugation in Eppendorf 5810R centrifuge (Hamburg, Germany) at 4000 rpm for 10 min. The cell free supernatants (CFSs) were lyophilized using a lyophilizer Virtis SP SCIENTIFIC sentry 2.0 (Warminster, EE.UU) and stored at −18 °C until the day of the analysis.

2.3. Antifungal activity of the LAB cell-free supernatant in solid medium

Kirby-Bauer assay, used to evaluate the antimicrobial activity of the LAB CFSs against *A. parasiticus* and *P. expansum*, was performed according to Varsha, Priya, Devendra, and Nampoothiri (2014) with some modifications. Lyophilized CFSs were reconstituted with 2 mL of sterile water and filtered with a 0.22 μm sterile nylon filter. For the agar well diffusion assay, 100 μL of the CFS were added to the wells (5 mm diameter) cut on PDA which was previously spread plated with *A. parasiticus* or *P. expansum* and kept at 26 °C for 2 days. A control composed of MRS broth treated in the same way of the CFSs was also prepared. The microorganisms were considered positive to the antimicrobial activity of the bioactive compounds if an inhibition zone of at least 8 mm wide was observed around the well.

2.4. Purification and peptides fractionation by gel filtration chromatography

The purification was carried out using the method described by Muhialdin, Hassan, Bakar, and Saari (2016) with some modifications. The lyophilized medium (400 mg) was dissolved in 2 mL deionized water and the peptides were fractionated using Sephadex G-25 size exclusion chromatography. The super fine resin can be used to separate peptides ranging between 1 and 5 KDa. The dry powder was swollen in boiling water for 3 h and the water was discarded and replaced with a separation buffer. The resin, which had been washed with 5 M HCl and degassed, was poured into the column (size 1.6 × 50 cm) and buffered with 0.2 M phosphate buffer pH 7. The flow rate was adjusted to 1 mL/min with a peristaltic pump and the end of the column was connected to a fraction collector FC 204 from Gilson (Middleton, USA) to recollect 80 fractions of 7 mL. The fractions were injected in the analytical liquid chromatography (LC) coupled to diode array detector (DAD) to identify the range of positive fractions to the presence of the peptides.

2.5. LC analyses of peptides

Peptide mixtures were analyzed by a LC system equipped with LC-7100 pump, autosampler L-2200 (20 μL loop) and a DAD L-7455 from Hitachi (Tokyo, Japan). Elution of the peptides was performed using an Aeris peptide XB-C18 (100 × 4.6 mm; 3.6 μM of ID, Phenomenex, Madrid, Spain) at 30 °C and a flow rate of 1 mL/min. The

mobile phase was composed of water (solvent A) and acetonitrile (solvent B) with 0.1% (v/v) trifluoroacetic acid (TFA). The elution gradient established was: 0 min-5% B; 10 min-35% B; 30 min-100% B; 40 min-100% B; 50 min-5% B (Brosnan, Coffey, Arendt, & Furey, 2014). The eluting peptides were monitored by measuring the absorbance at 214 nm.

2.6. Peptides isolation using semi-preparative LC

The fractions positive to the presence of peptides were injected for peptides isolation in a semi-preparative LC composed by a LC system equipped with LC-7100 pump, autosampler L-2200 (200 μL loop) and a DAD L-7455 from Hitachi (Tokyo, Japan). A semi-preparative Gemini C18 column (250 × 10 mm, 5 μM of ID) (Phenomenex, Madrid, Spain) was used and a mobile phase with water (solvent A) and acetonitrile (solvent B) containing 0.1% of TFA at flow rate of 3.0 mL/min. The elution gradient established was: 0 min-5% B; 10 min-35% B; 30 min-100% B; 40 min-100% B; 50 min-5% B. Peptides were detected at 214 nm. The end of the column was connected at a fraction collector FC 204 from Gilson (Middleton, USA).

The LC-purified peptides solutions were concentrated on a rotary evaporator at 35 °C, freeze-dried and stored at −80 °C before MALDI-TOF analysis.

2.7. MALDI-TOF analyses

Samples were dissolved in 0.1% (v/v) TFA and concentrated using the ziptip C18 pipette tips (Millipore, Billerica, MA). Peptide solution (1.0 μL) was mixed with the same volume of a-cyano-4-hydroxycinnamic acid matrix and spotted on a MALDI plate. MALDI-MS and MALDI-MS/MS were performed on an Applied Biosystems 4700 Proteomics Analyzer with TOF/TOF ion optics (Applied Systems, Foster City, CA). The mass spectrometer was operated in positive ion reflector mode with five spots of standard (ABI 4700 calibration mixture) for calibration. Mass spectra were obtained from each spot using 500 shots per spectrum. Tandem mass spectra were acquired by accelerating the precursor ions to 8 keV, selecting them with the timed gate set to a window of 3 Da, and performing CID at 1 keV. Gas pressure (air) in the CID cell was set at 0.2 μTorr. Fragment ions were accelerated to 14 keV before entering the reflector. The mass spectral data were submitted to a database search using the MASCOT program (Matrix Science, version 2.1).

2.8. Antifungal activity in liquid medium

The fungal strain of *A. parasitcus* and *P. expansum* were cultured in Potato Dextrose Broth (PDB) and antifungal activity was assessed with the method of Bolivar et al. (2011) with some modifications. Peptides previously isolated were dissolved in sterile water with concentrations ranging from 10 to 15,000 ppm.

For the antifungal tests, 9.8 mL of PDB were added to screw-capped tubes, followed by 0.1 mL of fungal inoculum (10^6 ufc/mL counted with a Neubauer chamber). After the inoculum was added, the tubes were treated with 0.1 mL of the peptide solution obtaining final concentration ranged from 0.1 to 150 ppm. Control groups contained 9.9 mL of PDB with water and 100 μL of inoculum. Experiments were performed in triplicate. The tubes were incubated for 48 h at 30 °C for Minimum Inhibitory Concentration (MIC) calculation and for 72 h for Minimum Fungicidal Concentration, (MFC) calculation at 30 °C under orbital shaking.

After that 100 μL of each tube were inoculated in a PDA Petri dishes and incubated at 30 °C during one week for the MIC and MFC calculation that are defined as the lowest concentration of an antimicrobial compound that will inhibit the visible growth of a microorganism after 48 and 72 h incubation. The cells colonies were also counted to perform the viability curve of the microorganism exposed at the different concentration of the peptides. All the experiments were performed in triplicate.

3. Results and discussion

3.1. Antifungal activity of the LAB cell-free supernatant in solid medium

The results related to the antifungal activity of the CFSs against *A. parasiticus* and *P. expansum* in solid medium of PDA are shown in Fig. 1. The analysis of the data demonstrate that the CFSs of *L. rhamnosus* CECT 278T, *L. johnsoni* CECT 289 and *L. plantarum* CECT 749 possess antifungal activity against the mycotoxigenic fungi tested. *L. plantarum* was the most effective with the largest inhibition zone, while *L. delbrueckii bulgaricus* CECT 4005 did not possess any antimicrobial activity against *A. parasiticus* and *P. expansum*. The control composed of MRS broth treated in the same way did not show evidence of any antifungal property against the mycotoxigenic fungi tested. Other authors investigated the antimicrobial properties of bioactive components produced by LABs. The results evidenced in our study are confirmed by the data obtained by Ryan, Bello, and Arendt (2008). These authors investigated the antifungal activity of two strains of *L. plantarum* fermented sourdoughs comparing their activity to that of a sourdough fermented by *L. sanfranciscensis* LTH2581 as well as to that of a chemically acidified dough and a dough containing antibiotics. *L. plantarum* fermeted sourdough showed the highest inhibitory activity. Valerio et al. (2009) tested fermentation products of 17 LAB strains against *Penicillium roqueforti* and *Aspergillus niger*. *Lactobacillus citreum*, *Weissella cibaria*, and *Lactobacillus rossiae* inhibited (>98%) the growth of *A. niger*. *L. plantarum* was the most effective against *P. roqueforti*. These results, in particular the data related to the antifungal activity of the bioactive compounds of *L. plantarum*, are in agreement with the data evidenced in our study. Ahmad Rather et al. (2013) screened 1400 bacteria isolated from different kimchi samples for their antifungal activity against *A. niger* by dual-culture agar plate assay. According to our study, the strain exhibiting the high antifungal activity was *L. plantarum* YML007 so that it was further screened against various pathogens showing the highest inhibition against *A. niger*, followed by *Aspergillus flavus, Aspergillus oryzae* and *Fusarium oxysporum*. Moreover biopreservative activity of *L. plantarum* YML007 was evaluated using dried soybeans and no fungal growth was observed in the soybeans treated with fivefold concentrated cell-free supernatant of *L. plantarum* YML007. Gupta and Srivastava (2014) studied the antifungal activity of 88 *L. plantarum* strains against *A. niger*, *A. flavus*, *Fusarium culmorum*, *Penicillium roqueforti*, *Penicillium expansum*, *Penicillium chrysogenum* and *Cladosporium* spp. Nine of these strains that strongly inhibited at least three moulds were further screened based on the antifungal properties of their cell-free supernatant. Even if none of the *L. plantarum* strain tested was able to completely inhibit the growth of the analyzed filamentous fungi, the maximum antagonistic effect was observed after 5 days of incubation in plates supplemented with CFS (12% v/v) from *L. plantarum* UFG 108 and *L. plantarum* UFG 121, in which the growth of *P. expansum* and *F. culmorum* was reduced about 50 and 60%, respectively. Finally an oat-based beverage obtained by fermentation with *L. plantarum* UFG 121 and contaminated with *F. culmorum* showed the best biopreservative effects without no differences in terms of qualitative features between not or contaminated samples.

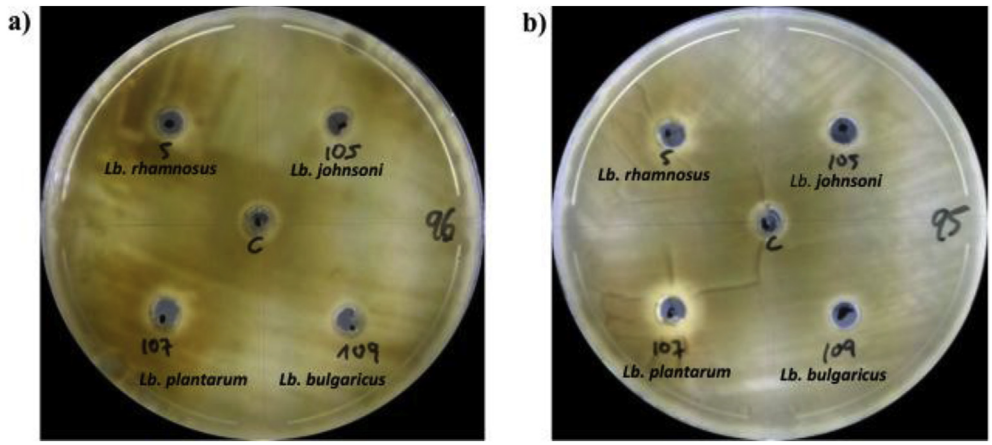

Fig. 1. Antifungal activity of the lyophilized fermented medium of MRS by prebiotic strains of *L. rhamnosus, L. johnsoni, L. plantarum,* and *L. delbrueckii bulgaricus* on the mycotoxigenic fungi a) *A. parasiticus* and b) *P. expansum*.

3.2. Isolation and identification of the antimicrobial peptides produced by L. plantarum in MRS broth

The gel filtration chromatography developed on G-25 fine sephadex was used for the fractionation of the bioactive peptides produced by *L. plantarum*. The fractions recollected by the purification process through the low pressure liquid chromatography were injected into the LC-DAD for the identification of the fractions positive to the presence of the peptides. Fraction three was particularly interesting for the presence of three peaks (Fig. 2a). They were purified by the technique of the LC-DAD using a semi preparative C18 column to recollect important amounts of the

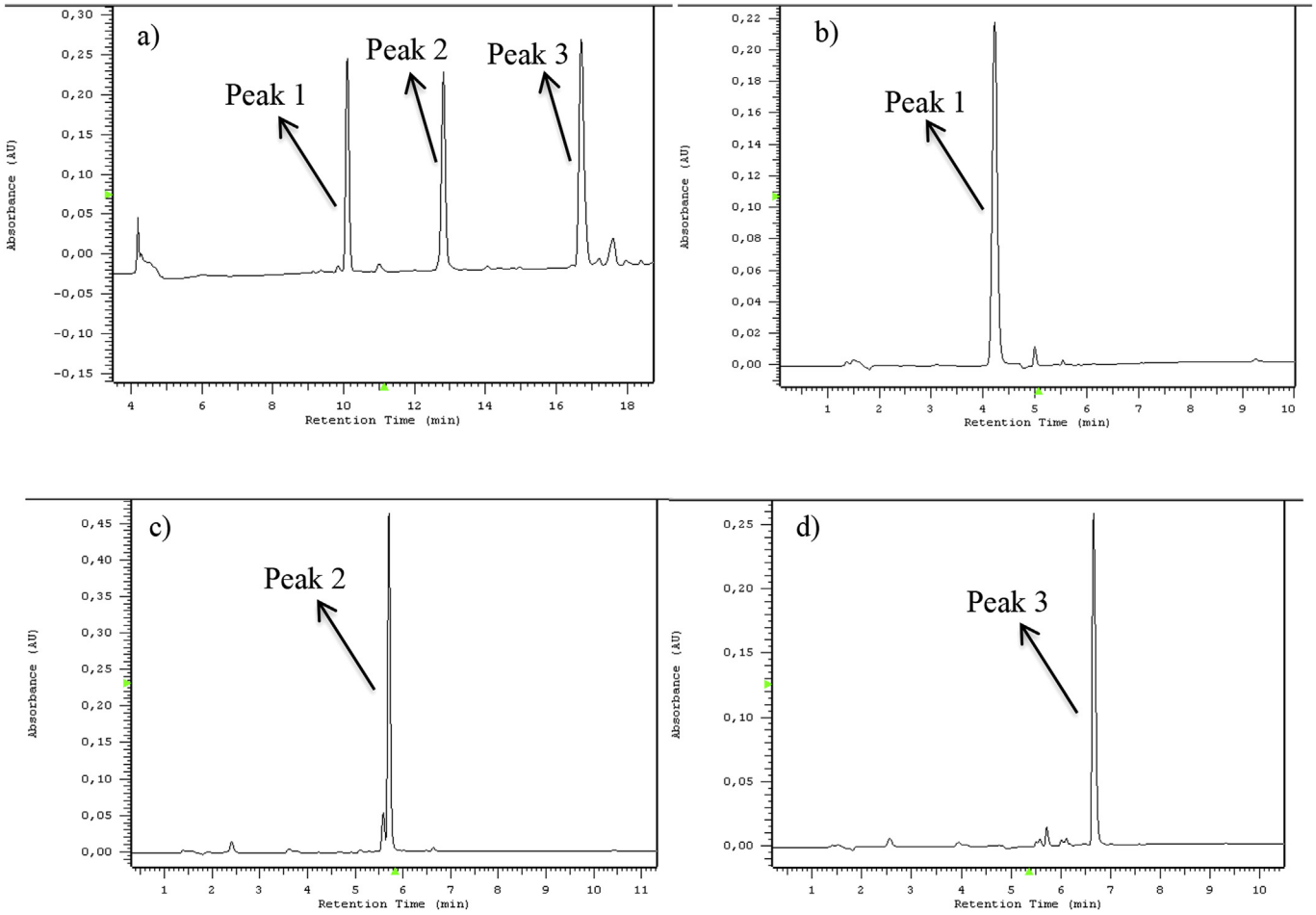

Fig. 2. a) Chromatogram of bioactive peptides produced by *L. plantarum* and analyzed with LC-DAD with an analytical column and **b) c)** and **d)** chromatograms of the peaks corresponding to the BP 1, 2 and 3 purified using a LC-DAD with a semipreparative approach column.

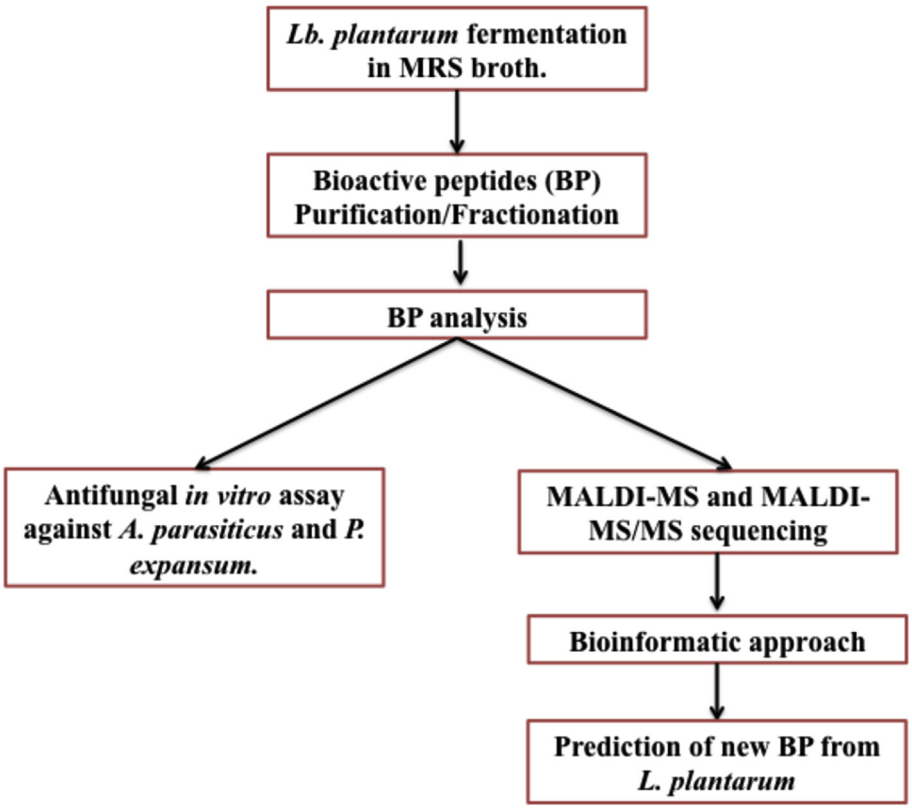

Fig. 3. Schematically approach for the study of the antifungal activity of the antimicrobial peptides produced by lactobacillus strains and of the mass spectrometry characterization of the new formed compounds through the MALDI-TOF.

Table 1
Purity, molecular weight and amino acid sequence of the bioactive peptides purified by a fermentation of *L. plantarum* in MRS broth.

Peak nº	Fraction purity (%)	Observed MS (Da)	Expected MS (Da)	Calculated MS	Delta	Peptide sequence
1	95%	1041.2600	1040.2527	1039.5186	0.73	SGADTTFLTK
2	96%	1019.3000	1018.2927	1018.6175	0.32	LVGKKVQTF
3	95%	995.1500	994.1427	993.5131	0.62	GTLIGQDYK

bioactive compounds, and then, their antifungal activity against *A. parasiticus* and *P. expansum* was studied (Fig. 3). As evidenced in Fig. 2b,c,d, each peak present in the fraction three was separated from the other compounds by the purification of the peptides (semipreparative chromatography approach) produced by the *L. plantarum* through a fermentation process in MRS medium. The analysis of the fractions purity evidenced that the semi preparative approach produced three different isolated compounds with 95, 96 and 95% of purity, respectively (Table 1).

Q-TOF-MS analysis indicated that the molecular mass of the peak 1 was 1041.26 Da. The observed molecular mass was in agreement with the calculated molecular mass of the peptide. The MS/MS analysis showed that the major sequence of the peak 1 was Ser-Gly-Ala-Asp-Thr-Thr-Phe-Leu-Thr-Lys (SGADTTFLTK).

The mass spectrometry analysis associated to the peak two, detected an observed molecular weight (MW) of 1019.30 Da and the MS^2 analysis of the fragment corresponding to the MW showed that the amino acidic sequence was composed by Leu-Val-Gly-Lys-Lys-Val-Gin-Thr-Phe (LVGKKVQTF).

Q-TOF-MS analysis associated to the peak three evidenced an observed MW that was in agreement with the calculated molecular mass of 995.15 Da. The MS^2 analysis of the ion corresponding to the MW evidenced an amino acidic sequence of the peak 1 of Gly-Thr-Leu-Ile-Gly-Gin-Asp-Tyr-Lys (GTLIGQDYK). The sequence of the three purified peaks, using low pressure gel filtration liquid chromatography associated to the LC with the semipreparative column was referred to a novel peptides, in accordance to the database available online (http://prospector.ucsf.edu; http://www.expasy.ch). The purified peptides were used as external standards for LC-DAD quantitative analysis. A stock solution was prepared in water and dilutions were made to cover a concentration range from 10 to 300 mg/L. The *L. plantarum* produced in the MRS medium 250.16 ± 4.7, 280.45 ± 7.2 and 246.87 ± 6.2 mg/L of the three bioactive peptides purified, respectively.

3.3. Antifungal activity of the peptides produced by L. plantarum against A. parasiticus and P. expansum

The use of natural sources of antimicrobial compounds has enormous potential due to their characteristics such as low toxicity and high specificity. Their mechanism of action is different against bacterial, fungal and animal cells. Bacterial cells have a layer rich in negatively charged phospholipids pointing toward the external environment, facilitating their interactions with peptides, most of which are positively charged. In contrast, animal cells are mainly composed of uncharged lipids in the outermost layer, and the

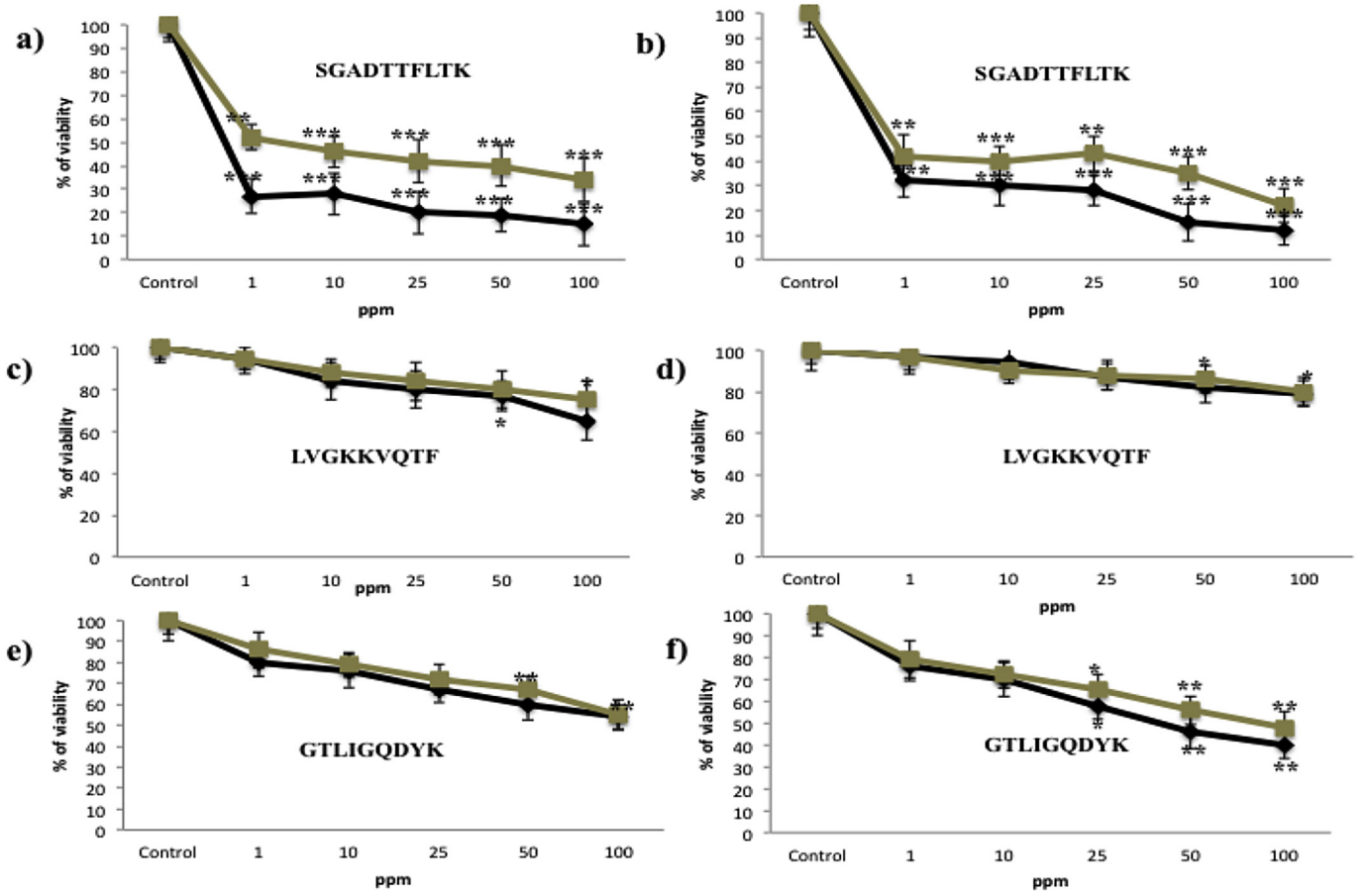

Fig. 4. Percentage of viability evidenced by *A. parasiticus* (a, c, d) and *P. expansum* (b, d, f) exposed at different concentration of the purified peptides at 24 h (black line) and 48 h (cinnamon line) incubation.

negatively charged regions are pointed toward the cytoplasm (Matsuzaki, 1999).

Antimicrobial peptides are widely distributed in nature and are essential to the immune system. They are the organism's first line of defense against colonization by exogenous microorganisms (Zasloff, 2002). More than 800 antimicrobial peptides have been described in plants and animals (Boman, 2003). Despite the great diversity in their primary structures, most antimicrobial peptides are similar as they are short amino acid chains composed primarily of cationic and hydrophobic amino acids (Dashper, Liu, & Reynolds, 2007). The low molecular weights of the peptide fractions, the resulting higher exposure of the amino acids and their charges, and the formation of small channels in the lipid bilayer promote interactions between the peptide and the membrane (Gómez-Guillén et al., 2010; Patrzykat & Douglas, 2005). The exact mechanisms of action for many antimicrobial peptides have not been well established (Dashper et al., 2007). Peptides with antimicrobial activity have also been identified in several protein hydrolysates.

In this study the antifungal activity of the three isolated peptides produced by *L. plantarum* was tested. The data related to the growth inhibition of *A. parasiticus* and *P. expansum*, after 48 and 72 h, induced by the exposure to different concentrations of the purified peptides are shown in Fig. 4. MIC and MFC could not be calculated since the peptides did not show the ability to reduce fungal growth ≥99% than the control at the concentrations tested. LVGKKVQTF and GTLIGQDYK peptides did not show significant antifungal activity against *P. expansum* and *A. parasiticus* if compared to the control. On the other side, a significant reduction of *A. parasiticus* and *P. expansum* growth was observed using all the concentrations of SGADTTFLTK peptide. In particular, *A. parasiticus* viability (%) was reduced of 73% and 42% after 48 and 72 h, respectively. Slightly lower reductions were obtained for *P. expansum*: 58% and 49% after the same incubation times. Several authors describe similar antimicrobial activity of peptides produced by LAB during fermentation. Russo et al. (2016) investigated the effect of antimicrobial peptides (AMPs LR14) produced by *L. plantarum* strain LR/14 against *A. niger*, *Rhizopus stolonifera*, *Mucor racemosus* and *Penicillium chrysogenum* by dual culture assay. All the four fungi were inhibited: the peptides inhibited both the spore germination and hyphal growth. In addition, fungal growth was inhibited in wheat seeds treated with AMPs LR14 even after a prolonged storage under laboratory conditions for 2.5 years. Muhialdin et al. (2016) reported that concentrations of 0.02 mg peptide/mL inhibited the fungal growth of *Aspergillus flavus*, *P. roqueforti* and *Eurotium rubrum* between 20 and 66%. Both peptide concentrations used in this study for the inhibition (%) of fungal growth resulted in the same order of magnitude as those obtained in our study. Wen, Philip, and Ajam (2016) studied the mode of action of a bacteriocin with low molecular weight, isolated from *L. plantarum* K25. This antimicrobial peptide, named plantaricin K25, exhibited a broad spectrum of inhibitory activity against both Gram-positive and Gram-negative bacteria. This bacteriocin showed to be a pore-forming bacteriocin capable of permeabilising the cytoplasmic membrane of targeted bacterial cells. It inhibited the growth of *B. cereus* cultivated in mul-kimchi with reduction of viable cell counts compared to the control sample. De Souza Barbosa et al. (2015) isolated two

bacteriocins, produced by *Lactobacillus curvatus*, with anti-*Listeria* activity from salami samples and evaluated their effectiveness in the control of *L. monocytogenes* during the fermentation step of salami manufacture. In particular, bacteriocin MBSa2 presented activity against 22 of 23 *L. monocytogenes* strains while the bacteriocin MBSa3 inhibited all 23 strains in addition to several other Gram-positive bacteria. Garofalo et al. (2012) studied two sourdough strains, *Lactobacillus rossiae* LD108 and *Lactobacillus paralimentarius* PB127 selected on the basis of their capacity to inhibit fungal growth, which was evidenced for the first time in their study. These authors observed that their antifungal properties against three cultures ascribed to *Aspergillus japonicus*, *Eurotium repens*, and *Penicillium roseopurpureum* were due to peptides identified as gluten proteolysis byproducts. Furthermore, the ability to prevent mold growth was demonstrated on bread for both strains, whereas *L. rossiae* LD108 also inhibited mold growth on panettone. Coda et al. (2011) investigated the antifungal activity of *Wickerhamomyces anomalus* and sourdough lactic acid bacteria to extend the shelf life of wheat flour bread. *Penicillium roqueforti* DPPMAF1 was used as the indicator fungus. Sourdough fermented by *Lactobacillus plantarum* 1A7 (S1A7) and dough fermented by *W. anomalus* LCF1695 (D1695) were selected and characterized. The water/salt-soluble extract of S1A7 was partially purified, and several novel antifungal peptides, encrypted into sequences of Oryza sativa proteins, were identified. The water/salt-soluble extract of D1695 contained ethanol and, especially, ethyl acetate as inhibitory compounds. Both water/salt-soluble extracts showed a large inhibitory spectrum, with some differences, toward the most common fungi isolated from bakeries. Bread making at a pilot plant was carried out with S1A7, D1695, or a sourdough started with a combination of both strains (S1A7-1695). Slices of the bread manufactured with S1A7-1695 did not show contamination by fungi until 28 days of storage in polyethylene bags at room temperature, a level of protection comparable to that afforded by 0.3% (wt/wt) calcium propionate.

4. Conclusions

In conclusion, the CFSs of *L. rhamnosus*, *L. johnsoni* and *L. plantarum* produced antifungal compounds during the fermentation against the mycotoxigenic fungi tested while *L. delbrueckii bulgaricus* was the only one that did not possess any antifungal activity against *A. parasiticus* and *P. expansum*. Liquid medium fermented by *L. plantarum* showed the highest inhibition activity, with the largest inhibition zone. Three peptides were purified from the culture medium fermented by *L. plantarum* and they were identified employing mass spectrometry MALDI-TOF: SGADTTFLTK, GTLIGQDYK and LVGKKVQTF. The LVGKKVQTF and GTLIGQDYK peptides identified have no observable antifungal activity. On the other side, the SGADTTFLTK peptide produced by *L. plantarum* during fermentation showed antifungal activity against *P. expansum* and *A. parasiticus* so that it could be a potential substitute to chemical preservatives in food.

Acknowledgments

The research was supported by the project for emerging research groups GV-2016-106 from the Generalitat Valenciana, by the pre PhD program of University of Valencia “Santiago Grisolia” and by the European Project (H2020-Research and Innovation Action) MycoKey “Integrated and innovative key actions for mycotoxin management in the food and feed chain” GA 678781.

References

Adebayo, C. O., & Aderiye, B. I. (2010). Antifungal activity of bacteriocins of lactic acid bacteria from some Nigerian fermented foods. *Research Journal of Microbiology, 5*, 1070–1082.

Bellei, B., Miguel, M., Mere Del Aguila, E. M., Silva, J. T., & Paschoalin, V. M. F. (2011). Purification of a bacteriocin produced by *Enterococcus faecium* and its effectiveness for preservation of fresh-cut lettuce. *Journal of Microbiology and Antimicrobials, 3*, 119–125.

Bolivar, P., Cruz-Paredes, C., Hernández, L. R., Juárez, Z. N., Sánchez-Arreola, E., Av-Gay, Y., et al. (2011). Antimicrobial, anti-inflammatory, antiparasitic, and cytotoxicactivities of Galium mexicanum. *Journal of Ethnopharmacology, 137*, 141–147.

Boman, H. G. (2003). Antibacterial peptides: Basic facts and emerging concepts. *Journal of Internal Medicine, 254*, 197–215.

Brosnan, B., Coffey, A., Arendt, E., & Furey, A. (2014). The QuEChERS approach in a novel application for the identification of antifungal compounds produced by lactic acid bacteria cultures. *Talanta, 129*, 364–373.

Brul, S., & Coote, P. (1999). Preservative agents in foods: Mode of action and microbial resistance mechanisms. *International Journal of Food Microbiology, 50*, 1–17.

Chen, H., & Hoover, D. G. (2003). Bacteriocins and their food applications. *Comprehensive Reviews in Food Science and Food Safety, 2*, 82–100.

Cizeikiene, D., Juodeikiene, G., Paskevicius, A., & Bartkiene, E. (2013). Antimicrobial activity of lactic acid bacteria against pathogenic and spoilage microorganism isolated from food and their control in wheat bread. *Food Control, 31*, 539–545.

Coda, R., Cassone, A., Rizzello, C. G., Nionelli, L., Cardinali, G., & Gobbetti, M. (2011). Antifungal activity of *Wickerhamomyces anomalus* and *Lactobacillus plantarum* during sourdough fermentation: Identification of novel compounds and long-term effect during storage of wheat bread. *Applied and Environmental Microbiology, 77*, 3484–3492.

Crowley, S., Mahony, J., & Van Sinderen, D. (2013). Current perspectives on antifungal lactic acid bacteria as natural bio-preservatives. *Trends in Food Science & Technology, 33*, 93–109.

Dal Bello, F., Clarke, C. I., Ryan, L. A. M., Ulmer, H., Schober, T. J., Strom, K., et al. (2007). Improvement of the quality and shelf life of wheat bread by fermentation with the antifungal strain *Lactobacillus plantarum* FST 1.7. *Journal of Cereal Science, 45*, 309–318.

Dalie, D. K. D., Deschamps, A. M., & Richard-Forget, F. (2010). Lactic acid bacteria - potential for control of mould growth and mycotoxins: A review. *Food Control, 21*, 370–380.

Dashper, S. G., Liu, S. W., & Reynolds, E. C. (2007). Antimicrobial peptides and their potential as oral therapeutic agents. *International Journal of Peptide Research and Therapeutics, 13*(4), 505–516.

De Souza Barbosa, M., Todorov, S. D., Ivanova, I., Chobert, J. M., Haertlé, T., De Melo, et al. (2015). Improving safety of salami by application of bacteriocins produced by an autochthonous *Lactobacillus curvatus* isolate. *Food Microbiology, 46*, 254–262.

De Vuyst, L., & Leroy, F. (2007). Bacteriocins from lactic acid bacteria: Production, purification, and food applications. *Journal of Molecular Microbiology and Biotechnology, 13*, 194–199.

Díez, L., Rojo-Bezares, B., Zarazaga, M., Rodríguez, J. M., Torres, C., & Ruiz-Larrea, F. (2012). Antimicrobial activity of pediocin PA-1 against Oenococcus oeni and other wine bacteria. *Food Microbiology, 31*(2), 167–172.

Gálvez, A., Abriouel, H., López, R. L., & Omar, N. B. (2007). Bacteriocin-based strategies for food biopreservation. *International Journal of Food Microbiology, 120*, 51–70.

Garofalo, C., Zannini, E., Aquilanti, L., Silvestri, G., Fierro, O., Picariello, G., et al. (2012). Selection of sourdough lactobacilli with antifungal activity for use as biopreservatives in bakery products. *Journal of Agricultural and Food Chemistry, 60*, 7719–7728.

Gerez, C. L., Torino, M. I., Rollan, G., & De Valdez, G. F. (2009). Prevention of bread mould spoilage by using lactic acid bacteria with antifungal properties. *Food Control, 20*, 144–148.

Gómez-Guillén, M. C., López-Caballero, M. E., López De Lacey, A., Alemán, A., Giménez, B., & Montero, P. (2010). Antioxidant and antimicrobial peptide fractions from squid and tuna skin gelatin. In E. L. Bihan, & N. Koueta (Eds.), *Sea by-products as a real material: New ways of application* (pp. 89–115). Kerala: Transworld Research Network Signpost.

Gupta, R., & Srivastava, S. (2014). Antifungal effect of antimicrobial peptides (AMPs LR14) derived from *Lactobacillus plantarum* strain LR/14 and their applications in prevention of grain spoilage. *Food Microbiology, 42*, 1–7.

Hassan, Y., Zhou, T., & Bullerman, L. B. (2015). Sourdough lactic acid bacteria as antifungal and mycotoxin-controlling agents. *Food Science and Technology International, 22*, 79–90.

Hugenholtz, J. (2013). Traditional biotechnology for new foods and beverages. *Current Opinion in Biotechnology, 24*, 155–159.

Laitila, A., Alakomi, H. L., Raaska, L., Mattila-Sandholm, T., & Haikara, A. (2002). Antifungal activities of two *Lactobacillus plantarum* strains against *Fusarium* moulds in vitro and in malting of barley. *Journal of Applied Microbiology, 93*, 566–576.

Mason, A. J., Moussaoui, W., Abdelrahman, T., Boukhari, A., Bertani, P., Marquette, A., et al. (2009). Structural determinants of antimicrobial and antiplasmodial activity and selectivity in histidine-rich amphipathic cationic peptides. *Journal of*

Biological Chemistry, 284, 119–133.
Matsuzaki, K. (1999). Why and how are peptide-lipid interactions utilized for selfdefense? Magainins and tachyplesins as archetypes. *Biochimica et Biophysica Acta, 1462*, 1–10.
Mauch, A., Dal Bello, F., Coffey, A., & Arendt, E. K. (2010). The use of *Lactobacillus brevis* PS1 to in vitro inhibit the outgrowth of *Fusarium culmorum* and other common *Fusarium* species found on barley. *International Journal Food Microbiology, 141*, 116–121.
Meroth, C. B., Walter, J., Hertel, C., Brandt, M. J., & Hammes, W. P. (2003). Monitoring the bacterial population dynamics in sourdough fermentation processes by using PCR-denaturing gradient gel electrophoresis. *Applied and Environmental Microbiology, 69*, 475–482.
Muhialdin, B. J., Hassan, Z., Bakar, F. A., & Saari, N. (2016). Identification of antifungal peptides produced by *Lactobacillus plantarum* IS10 grown in the MRS broth. *Food Control, 59*, 27–30.
Okkers, D. J., Dicks, L. M. T., Silvester, M., Joubert, J. J., & Odendaal, H. J. (1999). Characterization of pentocin TV35b, a bacteriocin-like peptide isolated from Lactobacillus pentosus with a fungistatic effect on Candida albicans. *Journal of Applied Microbiology, 87*, 726–734.
Parada, J. L., Caron, C. R., Medeiros, A. B. P., & Soccol, C. R. (2007). Bacteriocins from lactic acid bacteria: purification, properties, and use as biopreservatives. *Brazilian Archives of Biology and Technology, 50*, 521–542.
Patrzykat, A., & Douglas, S. E. (2005). Antimicrobial peptides: Cooperative approaches to protection. *Protein and Peptide Letters, 12*, 19–25.
Pawlowska, A. M., Zannini, E., Coffey, A., & Arendt, E. K. (2012). "Green preservatives": Combating fungi in the food and feed industry by applying antifungal lactic acid bacteria. *Advances in Food and Nutrition Research, 5*, 215–236.
Peyer, L. C., Axel, C., Lynch, K. M., Zannini, E., Jacob, F., & Arendt, E. K. (2016). Inhibition of Fusarium culmorum by carboxylic acids released from lactic acid bacteria in a barley malt substrate. *Food Control, 69*, 227–236.
Rather, A. I., Seo, B. J., Rejish Kumar, V., Choi, U., Choi, K., Lim, J., et al. (2013). Isolation and characterization of a proteinaceous antifungal compound from *Lactobacillus plantarum* YML007 and its application as a food preservative. *Letters in Applied Microbiology, 57*, 69–76.
Reis, J. A., Paula, A. T., Casarotti, S. N., & Penna, A. L. B. (2012). Lactic acid bacteria antimicrobial compounds: characteristics and applications. *Food Engineering Reviews, 4*, 124–140.
Rouse, S., Canchaya, C., & Van Sinderen, D. (2008). *Lactobacillus hordei* sp. nov., a bacteriocinogenic strain isolated from malted barley. *International Journal of Systematic and Evolutionary Microbiology, 58*, 2013–2017.
Rouse, S., Harnett, D., Vaughan, A., & Van Sinderen, D. (2008). Lactic acid bacteria with potential to eliminate fungal spoilage in foods. *Journal of Applied Microbiology, 104*, 915–923.
Russo, P., Arena, M. P., Fiocco, D., Capozzi, V., Drider, D., & Spano, G. (2016). *Lactobacillus plantarum* with broad antifungal activity: A promising approach to increase safety and shelf-life of cereal-based products. *International Journal of Food Microbiology*. http://dx.doi.org/10.1016/j.ijfoodmicro.2016.04.027.
Ryan, L. A., Bello, F. D., & Arendt, E. K. (2008). The use of sourdough fermented by antifungal LAB to reduce the amount of calcium propionate in bread. *International Journal of Food Microbiology, 125*, 274–278.
Sathe, S. J., Nawani, N. N., Dhakephalkar, P. K., & Kapadnis, B. P. (2007). Antifungal lactic acid bacteria with potential to prolong shelf-life of fresh vegetables. *Journal of Applied Microbiology, 103*, 2622–2628.
Schnurer, J., & Magnusson, J. (2005). Antifungal lactic acid bacteria as biopreservatives. *Trends in Food Science & Technology, 16*, 70–78.
Settanni, L., & Corsetti, A. (2008). Application of bacteriocins in vegetable food biopreservation. *International Journal of Food Microbiology, 121*, 123–138.
Smaoui, S., Elleuch, L., Bejar, W., Karray-Rebai, I., Ayadi, I., Jaouadi, B., et al. (2009). Inhibition of fungi and Gram negative bacteria by bacteriocin BacTN635 produced by *Lactobacillus plantarum* sp. TN635. *Applied Biochemistry and Biotechnology, 162*, 1132–1146.
Stoyanova, L. G., Ustyugova, E. A., & Netrusov, A. I. (2012). Antibacterial metabolites of lactic acid bacteria: Their diversity and properties. *Applied Biochemistry and Microbiology, 48*, 229–243.
Udhayashree, N., Senbagam, D., Senthilkumar, B., Nithya, K., & Gurusamy, R. (2012). Production of bacteriocin and their application in food products. *Asian Pacific Journal of Tropical Biomedicine*, S406–S410.
Valerio, F., Favilla, M., De Bellis, P., Sisto, A., De Candia, S., & Lavermicocca, P. (2009). Antifungal activity of strains of lactic acid bacteria isolated from a semolina ecosystem against *Penicillium roqueforti, Aspergillus niger* and *Endomyces fibuliger* contaminating bakery products. *Systematic and Applied Microbiology, 32*, 438–448.
Varsha, K. K., Priya, S., Devendra, L., & Nampoothiri, K. M. (2014). Control of spoilage fungi by protective lactic acid bacteria displaying probiotic properties. *Applied Biochemistry and Biotechnology, 172*, 3402–3413.
Wen, L. S., Philip, K., & Ajam, N. (2016). Purification, characterization and mode of action of plantaricin K25 produced by *Lactobacillus plantarum*. *Food Control, 60*, 430–439.
Yang, V. W., & Clausen, C. A. (2005). Determining the suitability of lactobacilli antifungal metabolites for inhibiting mould growth. *World Journal of Microbiology and Biotechnology, 21*, 977–981.
Zasloff, M. (2002). Antimicrobial peptides of multicellular organisms. *Nature, 145*, 389–395.