Lactobacillus brevis-based bioingredient inhibits *Aspergillus niger* growth on pan bread

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Abstract

Bread shelf life is generally compromised by fungi mainly belonging to *Aspergillus* and *Penicillium* genera, which colonise the surface of the product within few days from the production. The aim of this study was to select a *Lactobacillus brevis*-based bioingredient (LbBio) able to inhibit the growth of *Aspergillus niger* ITEM5132 on pan bread in order to prolong its shelf life. Four LbBio formulations, obtained by growing a selected *L. brevis* strain in a flour-based medium containing different carbon sources or acid precursors (fructose, LbBio1; fructose and maltose, LbBio2; α-chetoglutaric acid, LbBio3; short-chain fructooligosaccharides, LbBio4), were evaluated for their content of organic acids (lactic, acetic, propionic, phenyllactic, 4-hydroxy-phenyllactic, valeric, isovaleric acids). The LbBio formulations were applied in yeast-leavened bread during bread-making trials and the resulting products were inoculated after baking with *A. niger* spore's suspension and the fungal growth was monitored during storage (25°C for 6 days). The formulation showing the highest inhibitory activity was separated by ultra-filtration method, and whole and fractions obtained were evaluated for their *in vitro* activity. The fraction showing the highest activity was further separated by gel-filtration and the resulting products were investigated for their protein content and *in vitro* inhibition.

The results from the bread-making trials performed using different formulations of LbBio showed a delay in fungal growth (1 day) respect to the bread not containing the bioingredient (control) or including calcium propionate (0.3% w/w). The formulation LbBio2, prepared with fructose and maltose 1% (w/vol), contained the highest amount of total organic acids, including phenyllactic and hydroxyl-phenyllactic acids, and reduced the visual spoilage of bread. This formulation was separated by ultra-filtration and fractions containing metabolites with molecular weight higher than 30 kDa showed high inhibitory effect in the *in vitro* assay. In particular, the microfluidic analysis highlighted the presence of a protein with a molecular weight of 56 kDa only in the active fraction. Further studies have to be done in order to identify the protein involved in the antifungal activity.

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Introduction

Bread is one of the most important staple foods in the world and it is generally considered as a perishable commodity. The quality of bakery products is compromised by the contamination of raw materials by bacteria, yeast and moulds (Lavermicocca *et al.*, 2003; Valerio *et al.*, 2012; Legan and Voysey, 1991). Mould growth is the most common form of microbial spoilage in bread leading to huge economic losses (Legan, 1993) as well as reduced safety for consumers due to the production of toxic (mycotoxins) and allergenic (Visconti and Bottalico, 1983; Filtenborg *et al.*, 1996; Latgè and Paris, 1991) compounds. Fungal spoilage of wheat bread is mainly due to *Penicillium* spp. and other fungi belonging to *Aspergillus* and *Cladosporium* genera.

In the course of the time, different strategies were proposed to preserve bread from moulds proliferation: addition of chemical compounds like benzoate, propionate and sorbate (Guynot *et al.*, 2005) or preservation by using modified atmosphere (Karin and Per, 2005).

The European Directive on preservatives (1995/2/EC; European Commission, 1995) regulates and approves the use of sorbate (0.2%, w/w), propionate (0.3%, w/w) and ethanol (up to 2%, w/w), as antifungal preservatives but their inhibitory efficacy depends on the fungal species contaminating the product.

Consumer's demand for reduced use of chemical preservatives prompted the need for alternative preserving strategies such as the use of bacteria naturally occurring in food. Actually, lactic acid bacteria (LAB) represent ideal candidates for this application, since their recognised *generally regarded as safe* status and their qualified presumption of safety status in the EU. Recently, LAB and their antimicrobial metabolites (such as organic and fatty acids, hydrogen peroxide, diacetyl and bacteriocins) are used in the bakery process to provide a natural preservation by reducing the presence of chemical preservatives (Lavermicocca *et al.*, 2000, 2003; Ström, 2005; Dal Bello *et al.*, 2007; Gerez *et al.*, 2009). Furthermore, the use of LAB improves the physico-chemical and textural quality of the product by reducing the pH value of the dough and increasing the total titratable acidity

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(Wehrle *et al.,* 1997; Katina *et al.*, 2002; Komleni *et al.*, 2010; Valerio *et al.*, 2014). In the latter study a *L. brevis* strain was selected to produce bioingredients efficient in improve yeast-leavened bread shelf life and its textural properties without affecting the leavening performance of *S. cerevisiae* (Valerio *et al.*, 2014).

The genus *Lactobacillus* was frequently involved in the antifungal activity of LAB as recently reported by some authors (Stiles, 1996; Gerez *et al.,* 2009, 2013; Ryan *et al.*, 2011; Ryu *et al.*, 2014; Black *et al.*, 2013) and the majority of identified antifungal substances are organic acids, hydroxy fatty acids, hydrogen peroxide, reuterin and proteinaceous compounds. Lactic and acetic acids are the main products of the fermentation of carbohydrates by LAB that generally diffuse through the membrane of the target organisms (Axelsson, 1990) in their hydrophobic undissociated form and then reduce cytoplasmatic pH and stop their metabolic activities. Other acidic molecules recognised as antifungal compounds and produced by LAB are propionic, acetic and phenyllactic (PLA) acids. In particular, it was hypothesised that PLA may act in a synergy way with other antifungal substances also of proteinaceous nature (Lavermicocca *et al.,* 2000; Magnusson and Schnürer, 2001; Ström *et al.,* 2002; Gerez *et al.,* 2009, 2013; Ryan *et al.*, 2011). Actually, Magnusson and Schnürer (2001) reported that the antifungal metabolite produced by *L. coryniformis* subsp. *coryniformis* Si3 strain was a highly heat stable, small peptide of approximately 3 kDa. Thus, probably, the LABs' inhibitory effect was not only due to the organic acid compounds but in agreement with other authors (Corsetti *et al.*, 1998; Niku-Paavola *et al.,* 1999; Ryan *et al.*, 2011; Gerez *et al.*, 2013) to synergistic activities between organic acids and peptides from the lactic acid bacterial fermentation.

The aim of the present study was to investigate the efficacy of metabolites produced by a selected *L. brevis* strain in yeast-leavened bread and the chemical nature of antifungal compounds.

Materials and methods

Culture conditions and *Lactobacillus brevis*-based bioingredient preparation

Lactobacillus brevis ITM18 was isolated from sourdough and belonged to the Collection of Institute of Sciences of Food Production - National Research Council of Bari. For long-term storage, stock cultures were prepared by mixing 8 mL of a culture with 2 mL of Bacto glycerol (Difco, Becton Dickinson Co., Sparks, MD, USA) and freezing 1 mL portions of this mixture at –80°C. Culture was stored frozen (–80°C) in MRS broth (Oxoid Ltd., Basinstoke, UK) supplemented with 20% Bacto glycerol (Difco) and subcultured twice before use.

The bioingredient (LbBio) was prepared inoculating 1% vol/vol of an overnight (37°C, 150 rpm) *L. brevis* ITM18 culture in a flour-based medium (FBM, pH 6.2). Four LbBio compositions were obtained by a mixture of wheat flour (100 g), water (500 mL): i) with 1% (w/vol) fructose (Difco) (LbBio1); ii) with 1% (w/vol) fructose and 1% (w/vol) maltose (Difco) (LbBio2); iii) with 0.1% (w/vol) α-chetoglutaric acid (Sigma Aldrich, Milan, Italy) (LbBio3); iv) with 1% (w/vol) short-chain fructooligosaccharides (scFOS, Sigma) (LbBio4). After incubation, each LbBio compositions were centrifuged (10,000 rpm, 10 min, 4°C) and the supernatants were further filtered with filter paper (Whatman N.4). As a control FBM incubated in the same conditions (37°C, 150 rpm, 18 h) but not inoculated with the *L. brevis* strain, was used. Each LbBio or the FBM were combined, instead of water amount, with ingredients of wheat pan bread.

Fungal spore production

The strain *Aspergillus niger* ITEM5132 was isolated from bread and belonged to the ITEM Collection of Institute of Sciences of Food Production - National Research Council of Bari. Three-days old spores of *A. niger* ITEM5132 were plated on potato dextrose agar (Difco) at 25°C with light/dark cycle (12/12 h), collected and suspended in Triton X-100 0.05% (vol/vol) (Lavermicocca *et al.,* 2003). Spores were counted in the Thoma chamber and 1 mL of the spore suspension (10^3 spores/mL) was nebulised on pan bread samples after baking.

Organic acid quantification in bioingredients

The analysis of organic acids content (lactic, acetic, propionic, phenyllactic PLA, 4-hydroxy-phenyllactic OH-PLA, valeric, isovaleric acids) in the bioingredients and in FBM, were performed as reported in Valerio *et al.* (2014). Each bioingredient was centrifuged (8422 × g, 10 min) and the supernatant was freeze-dried, resuspended in high-performance liquid chromatography (HPLC) mobile phase (0.005 mol/L H_2SO_4, Fluka, Deisenhofen, Germany) and passed through a micro-concentrator (Ultracel-3 k, Amicon, Danvers, MA, USA) with a molecular-mass cut-off of 3000 Da, by centrifugation (7000 × g, 2°C, 1 h). Solutions were loaded onto the column. The analysis of organic acids was performed by HPLC (AKTABasic10, P-900 series pump, Amersham Biosciences AB, Uppsala, Sweden), using a Rezex ROA-organic acid H+ (8%) column (7.80×300 mm, Phenomenex, Torrance, CA, USA) and a 3-channel UV detector (Amersham Biosciences 900) set at 210 and 220 nm. The mobile phase was pumped at a flow rate of 0.6 mL/min through the column heated to 70°C. Quantification of the organic acids was performed by integrating calibration curves obtained from the relevant standards. The quantification limits were 21.211 mM for lactic, 30.376 mM for acetic, 1.984 mM for propionic, 4.538 mM for valeric, 1.286 mM for isovaleric, 0.011 mM for PLA and 0.009 mM for OH-PLA acids.

Bread-making trials

The bread-making trials were performed to evaluate the efficacy of different LbBio compositions to inhibit the *A. niger* ITEM5132 growth on bread surface. Breads were made by kneading 350 g wheat flour, 5 g margarine, 5 g sugar, 10 g fresh baker's yeast (*Saccharomyces cerevisiae*) and 210 mL of tap water or of LbBio, or of FBM or of a calcium propionate water solution (0.3% w/w flour), in a mixer-cooking machine (Princess® Home Breadmaker, type 1936; Princess Household Appliance BV, Breda, Netherlands). Breads containing water or the FBM or calcium propionate were used as controls (Control 1, Control 2 and Control 3, respectively). The bread-making process included the following steps: 1st kneading (10 min), 1st leavening (20 min), 2nd kneading (15 min), 2nd leavening (20 min), forming (30 s), last leavening (55 min), baking (60 min). The pH values after cooking were registered with pH meter for each bread that was in sterile conditions and stored in polyethylene bags after that 1 mL of *A. niger* ITEM5132 spores suspension (10^3 spores/mL) was nebulised on bread surface. Finally, loaves were stored at 25°C and daily examined until the appearance of *A. niger* growth on Control 1 bread. The bread-making trial *Test 1* was performed to evaluate the efficacy of different LbBio compositions (LbBio1, LbBio2, LbBio3, LbBio4) to inhibit the *A. niger* ITEM5132 growth on bread surface. Furthermore, a selected *L. brevis* bioingredient formulation was subjected to a ultrafiltration separation and whole and selected fractions were evaluated in bread-making trials (*Test 2*). In each test, water (Control 1), FBM (Control 2) and calcium propionate (Control 3) were used as controls.

Lactobacillus brevis-based bioingredient by ultrafiltration and gel filtration

The selected LbBio2 was subjected to ultrafiltration by using

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Labscale™ TFF System and the microfiltration membranes Pellicon® XL Device (Millipore Corp., Billerica, MA, USA). In particular, the bioingredient was separated in the following order: Biomax-5 (5 kDa), Biomax-30 (30 kDa) and Biomax-50 (50 kDa). The ultrafiltration products obtained from each microfiltration membrane were: permeate fractions (P5, P30, P50) and retain fractions (R5, R30, R50). All fractions were used in the *in vitro* agar assay inhibitory test.

The selected ultrafiltrated product (R30) was further separated by gel-filtration method. The R30 fraction was lyophilised, resuspended in phosphate buffer (50 mM-0.15 M NaCl, pH 7) and separated on a Superose 12 HR 10/30 column (GE Healthcare Bio-Sciences AB) with a chromatographic system (AKTA Basic 10, P-900 series pump) using a 500 μL loop and a UV detector (Amersham Biosciences 900) set at 280 nm and 220 nm. One ml of each fractions was collected. All fractions obtained by gel filtration were tested in *in vitro* microdilution test.

Preliminary characterisation of antifungal compounds by microfluidic electrophoresis

R30 fractions from gel filtration were analysed for the protein content by the Bradford assay (Bradford, 1976). The calibration curve was obtained using defined concentration of the bovine serum albumin [bovine serum albumin (BSA), fraction 5] (Bio-Rad) solution and its dilutions, as standards. Briefly, 10 μL of each samples were diluted in 790 μL of distilled water and 200 μL of Bradford reagent. The protein concentration of samples was calculated with spectrophotometric method, measuring the absorbance at 595 nm and interpolated values obtained with standard curve of BSA. Only fractions containing proteins, concentrated by micro-concentrator at molecular-mass cut-off of 3000 Da (Ultracel 3-K, Amicon, Danvers, MA, USA), were analysed by sodium dodecylsulfatepolyacrylamide gel electrophoresis (SDS-PAGE). Protein sizing and analysis were done using an Agilent 2100 Bioanalyzer (Agilent Technologies GmbH, Waldbronn, Germany) based on the microfluidic technology that minimises chemical and analytical procedures and shortens analysis time, automating the whole process. Chip-based protein analysis was performed according to the manufacturer's protocol using a Protein 200 Plus LabChip kit and dedicated Protein 200 Plus assay software (Expert). Proteins were separated electrophoretically and data were translated into individual electropherograms (molecular weight against fluorescence units). To obtain sizing of proteins, a Ladder, consisting of 11 different proteins with a lower limit marker at 6 kDa and an upper limit marker at 210 kDa, was run on each chip to provide an internal calibration. Upper and lower markers are also incorporated in each unknown sample for direct comparison against the sizing ladder.

In vitro inhibition assays

To assess the inhibitory efficacy of ultrafiltrated and R30 fractions, the *in vitro* agar assay and the microdilution test were applied.

The *in vitro* agar test was performed in bread extract broth (BEB) agar. This medium was obtained as described by Pepe *et al.* (2003) using bread (Control 1) without preservative produced in laboratory. Briefly, 100 g of bread and 350 mL of distilled water were homogenised in stomacher Lab-Blender 400 for 2 min; the suspension was then centrifuged (10,000 rpm, 10 min), filtered (Whatman N.1) and the pH was adjusted to 6.8 with NaOH 1 M. BEB was supplemented with Agar Technical (16 g/L) (Difco) and sterilised (121°C, 15 min). Each LbBio and ultrafiltrated samples (200 μL) were plated on BEB agar. As control, HCl-acidified water (CoI, pH 3.34) was used. Once the samples were dried on the plate, 100 μL of *A. niger* ITEM5132 spore suspension (about 10^2 spore/mL) were spread plated and plates were incubated at 25°C for 48 h. The test was performed in duplicate. After incubation, germinating spores were counted at the stereoscope.

The inhibitory activity was reported as arbitrary units (AU), calculated as follow:

$$1\ AU = (UFC_{Co\,I} - UFC_S) = 100\ UFC \quad (1)$$

were $UFC_{Co\,I}$ was the number of germinating conidia counted on plates containing HCl-acidified water and UFC_S was the number of germinating conidia on the plates containing samples. The activity percentage of each sample was calculated in comparison with activity obtained using LbBio2. The microdilution test was used to evaluate the antifungal activity of R30 fractions obtained by gel filtration. The assay was performed in sterile, disposable, multiwell microdilution plates (96 wells, IWAKI, Scitech Div., Asashi Techno Glass, Tokyo, Japan) as reported in Lavermicocca *et al.* (2003). Briefly, 50 μL of each fraction were added to BEB inoculated with fungal spores (about 10^4 spores/mL) and not inoculated (blank). Inoculated wells were prepared in quintuplicate, and blank was prepared in triplicate. All microdilution plates were incubated in a humid chamber at 25°C for 72 h. Fungal growth was measured by determining the optical density at 580 nm every 24 h with a spectrophotometer (LabsystemMultiskan MS, version 3.0, type 352). The inhibition percentage was calculated in comparison with fungal growth in control wells.

Results

Organic acids in *Lactobacillus brevis*-based bioingredient formulations and antifungal efficacy in bread-making trials

No trace of acetic, propionic, valeric and OH-PLA acids was found in the FBM but all bioingredients contained lactic, propionic, valeric and PLA acids (Table 1). The concentration of lactic and acetic acid, PLA and OH-PLA produced by the selected *L. brevis* ITM18 strain in the bioingredient containing fructose and maltose as carbon source, named LbBio2, was found to be significantly higher (P>0.05) than in the other

Table 1. Organic acid content in different *L. brevis*-based bioingredient compositions.

	Acid concentration (mM)							
	Lactic	Acetic	Propionic	Valeric	Isovaleric	PLA	OH-PLA	Total content
FBM	49.499	-[a]	-[a]	-[a]	1.36	0.021	-[a]	50.880
LbBio1	71.451	-[a]	7.153	17.407	2.23	0.029	-[a]	98.270
LbBio2	71.644	58.340	10.969	15.363	5.48	0.051	0.032	161.879
LbBio3	56.320	-[a]	8.558	8.356	-[a]	0.024	-[a]	73.258
LbBio4	66.678	-[a]	11.710	14.926	5.32	0.033	-[a]	98.667

PLA, phenyllactic acid; OH-PLA, 4-hydroxy-phenyllactic acid; FBM, flour-based medium; LbBio, *L. brevis*-based bioingredient composition. [a]<LOQ. The quantification limits (LOQ) were 21.211 mM for lactic, 30.376 mM for acetic, 1.984 mM for propionic, 4.538 mM for valeric, 1.286 mM for isovaleric, 0.011 mM for PLA and 0.009 mM for OH-PLA acids.

bioingredients. In particular only in this composition *L. brevis* produced acetic and OH-PLA acids. Moreover in LbBio2 a greater concentration of total acid content than other bioingredients was observed. Lactic acid was produced at concentration by 49.499 mM (FBM) to 71.644 mM (LbBio2), instead the acetic acid was produced only in LbBio2 (58.340 mM). Both PLA and OH-PLA were found to be produced only in LbBio2. The organic acid content in FBM not inoculated with *L. brevis* was probably due to the presence of an endogenous microbial population in the flour used to produce the medium. In bread-making *Test 1* the four bioingredients (LbBio1, LbBio2, LbBio3, LbBio4) were compared to Control bread 1 and 2 containing water or calcium propionate (0.3%, w/w flour), respectively. All tested bioingredients allowed to obtain bread pH values lower than control breads (Table 2). A 1 day delay (respect control bread 1) in the appearance of *A. niger* growth was observed in all bread samples containing bioingredients, showing an inhibitory efficacy comparable to that of propionate (*data not shown*). In particular, LbBio2 caused a delay of fungal growth and a lower fungal colonisation indicating an interesting preserving ability and a thermal stability of the active metabolites during the bread cooking.

Preliminary characterisation of antifungal compounds

Fractions of LbBio2 were separated by ultrafiltration with membrane cut-off of 5 kDa,30 kDa and 50 kDa to isolate the antifungal compounds. Table 3 showed that the permeate P5, containing molecules with molecular mass lower than 5 kDa, not influenced *A. niger* growth, instead the antifungal efficacy occurred in R5 fraction, characterised by compounds with weight >5 kDa; therefore R5 was further ultrafiltrated with membrane cut-off of 30 kDa. The resulting R30 fraction was ultrafiltrated with membrane cut-off of 50 kDa. Fractions R30, P30, R50 e P50 showed a distributed anti-fungal activity. In particular, R30 and R50 were efficient in inhibiting *A. niger* growth, indicating that active molecules of LbBio2 had a molecular mass >30 kDa. Moreover, preliminary results showed that antifungal activity was not modified after thermal treatment of fractions, confirming a high thermal stability (*data not shown*).

The most active fraction (R30, Table 3) was used in comparison with unfractionated LbBio2 in a bread-making trial (*Test 2)*. The selected fraction (Figure 1D) confirmed its efficacy showing preserving performance similar to that of propionate-containing bread (Figure 1B).

Gel-filtration method was applied to R30 and fractions were further analysed by SDS-PAGE gel electrophoresis and tested for the antifungal activity. Fractions from 7 to 24 contained proteins and were tested by microdilution test on *A. niger*. The antifungal activity was observed in fractions from 7 to 13 and from 21 to 24 at percentage ranging from 47.4% to 100% (Table 4). In particular, fractions from 8 to 10 showed 100% inhibition of fungal growth, while the antifungal activity of fractions from 14 to 20 was not observed. The remaining fractions showed intermediate values of inhibitory activity (Table 4).

Furthermore, the SDS-PAGE microfluidic electrophoresis performed on fractions from 10 to 24,indicated that the active fractions (10, 11, 20, 21, 22) contained proteins at molecular mass ranging from 15 to 63 kDa. In particular, 29% of the protein content of the active sample R30 was represented by a protein with molecular mass of about 56 kDa.

Discussion and conclusions

Several strategies have been developed to extend the bread shelf-life generally compromised by fungal contamination which causes economic losses to baking industries and improve its quality (Legan and Voysey, 1991; Valerio *et al.*, 2014). The increased interest in biopreser-

Table 2. pH values of bread samples containing the four *L. brevis*-based bioingredient compositions (LbBio), water (Control 1) or flour-based medium (Control 2) or calcium propionate (0.3% w/w flour, Control 3), instead of the LbBio.

Bread sample	Bread pH
LbBio1	4.59
LbBio2	4.58
LbBio3	4.57
LbBio4	4.51
Control 1 (water)	5.70
Control 2 (FBM)	5.10
Control 3 (propionate)	5.17

LbBio, *L. brevis*-based bioingredient composition; FBM, flour-based medium.

Table 3. Inhibitory efficacy of *L. brevis*-based bioingredient fractions obtained by ultrafiltration against *A. niger* ITEM5132 growth. The inhibitory activity is reported as arbitrary units. The activity percentage of each sample was calculated in comparison with activity obtained using LbBio2.

Sample	*A. niger* ITEM5132	
	AU*	Inhibition (%)
LbBio2	3051	-
R5	2269	74.4
P5	0	0
R30	3051	100
P30	1080	35.3
R50	1852	61
P50	800	26.2

AU, arbitrary units; LbBio, *L. brevis*-based bioingredient composition. *AU were obtained using the following formula: 1 AU = (UFC_{Co} I – UFC_S) = 100 UFC; UFC_{Co} I : number of germinating conidia counted on plates containing HCl–acidified water and UFC_S was the number of germinating conidia on the plates containing samples.

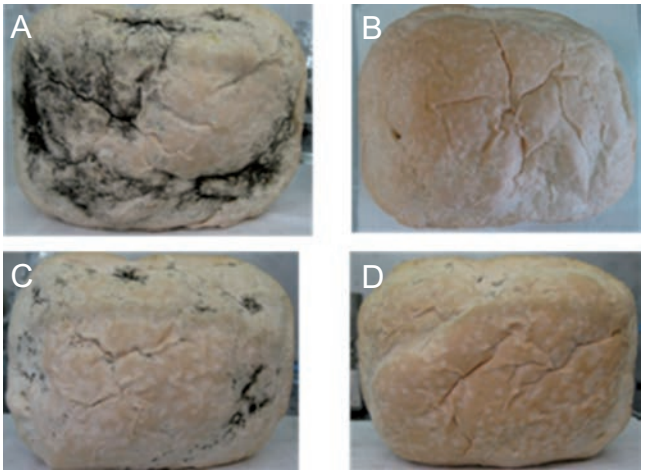

Figure 1. *A. niger* ITEM5132 growth on the surface of yeast-leavened pan bread containing water (A) and calcium propionate (0.3% w/w flour) (B) as controls and containing the selected *L. brevis*-based bioingredient LbBio2 (c) and its ultrafiltrated fraction R30 (D). Loaves were incubated at 25°C and examined after 6 days.

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vation of food systems has recently led to the development of new natural antimicrobial compounds having different origins. A variety of systems to prevent food spoilage have been investigated mainly because of the consumers demand for natural products instead of chemicals such as food preservatives (Gray and Bemiller, 2003). The current study confirmed the antifungal properties of LAB bioingredients indicating a synergistic role of organic acids (mainly phenyllactic) and proteinaceous compounds in their efficacy, as also previously suggested (Lavermicocca *et al.*, 2000; Gerez *et al.,* 2010; Black *et al.*, 2013; Ryu *et al.*, 2014; Ryan *et al.,* 2011). In addition the influence of the carbon source or acid precursors on the organic acid production by a selected *Lactobacillus brevis* strain was demonstrated. In particular, the presence of fructose and maltose in a flour-based medium allowed to obtain a bioingredient (LbBio2) with interesting antifungal performance observed both in *in vitro* and in bread-making trials. The influence of the carbon source on the antifungal performances of lactic acid bacteria has also been reported by Rouse *et al.* (2008) who demonstrated that the antifungal activity of the LAB strains tested (*L. plantarum, Weissella confusa, W. cibaria, Pediococcus pentosaceus*) against *Penicillium notatum* varied depending from the carbon source used. Authors found a relationship between the growth conditions and the antifungal properties which were only partially due to the organic acid content. Actually, the LbBio2 was characterised by the simultaneous presence of both PLA and OH-PLA, by the higher total organic acid content and the lower pH value, thus confirming the role of acid compounds in the antifungal efficacy as also demonstrated by Valerio *et al*. (2009). Authors identified three strains (*Weissella cibaria, Leuconostoc citreum* and *Lactobacillus rossiae*) whose fermentation products showed a strong inhibitory activity against *A. niger* isolated from bakery products and were characterised by low pH values and high content of lactic and acetic acids. As a results, the pH dependent effectiveness of many anti-fungal compounds (Suhr and Nielsen, 2004), was also observed for LbBio2 bread produced in *Test 1* which showed to the lower bread pH and an appreciable antifungal activity. When the selected LbBio2 was separated by ultrafiltration to deeper investigate the possible presence of other active metabolite other than organic acids, interestingly the activity was retained in the fraction containing molecules with size higher than 30 kDa. These results suggested that the antifungal activity observed in LbBio2 could be attributed to the pool of metabolites produced by the *L. brevis* strain including proteinaceous compounds in agreement with other authors (Corsetti *et al.*, 1998; Niku-Paavola *et al.,* 1999; Ryan *et al.*, 2011; Gerez *et al.*, 2013; Atassanova *et al*., 2003; Falguni *et al*., 2010). In particular, Falguni *et al.* (2010) selected a *L. brevis* strain for its antifungal activity and they demonstrated the proteinaceus nature of the active substances and their molecular weight ranged from 1 kDa to 5 kDa. Actually, the involvement of small peptides in the inhibition of mould growth has been widely demonstrated (Schnürer and Magnusson, 2005) while there are few evidences on the role of larger protein compounds in the antifungal activity of LAB. However, Atanassova *et al.* (2003) demonstrated the production of a protein with molecular mass of about 45 kDa by a *L. paracasei* subsp. *paracasei* strain which showed bactericidal and fungistatic activities. In the current study, results obtained by the SDS-PAGE microfluidic analysis indicated the presence of a protein with molecular mass of about 56 kDa acting in a synergistic way with organic acids. The use of bioingredients to prevent the microbial contamination can be an efficient approach to limit the use of chemical preservatives also in yeast-leavened bread within the hurdle technologies. Further studies are required to identify the metabolites involved in the anti-fungal activity of *L. brevis* ITM18 and to examine in depth the antifungal synergistic activities between organic acids and peptides.

Table 4. *Aspergillus niger* ITEM5132 growth inhibition caused by R30-LbBio2 fractions obtained by gel filtration.

Sample	Growth inhibition (%)
F7	99.6
F8	100
F9	100
F10	100
F11	75.8
F12	98.3
F13	31.08
F14	-
F15	-
F16	-
F17	-
F18	-
F19	-
F20	-
F21	47.4
F22	51.0
F23	49.7
F24	60.1

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