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ORIGINAL RESEARCH

A natural strategy to improve the shelf life of the loaf bread against toxigenic fungi: The employment of fermented whey powder

CARLOS LUZ,[1]* LORENA RODRIGUEZ,[2] RAFFAELE ROMANO,[3] JORGE MAÑES[1] and GIUSEPPE MECA[1]

[1]*Faculty of Pharmacy, Laboratory of Food Chemistry and Toxicology, University of Valencia, Av. Vicent Andrés Estellés s/n, 46100 Burjassot,* [2]*Instituto Tecnológico del Plástico (AIMPLAS), València Parc Tecnològic, 46980 Paterna, Spain and* [3]*Department of Agriculture, University of Napoli Federico II, Via Università, 100, 80055 Portici (Napoli), Italy*

Whey powders are used as food ingredients in many applications, from bakery goods, soups and sauces to baby food. The objective of the study was to evaluate the antifungal property of a whey-based medium (WM) fermented by lactic acid bacteria. The antifungal activity of the WM was evaluated using antifungal tests on solid and liquid media. MIC and MFC ranged from 15.6 to 250 mg/mL and 62.5 to 250 mg/mL, respectively. Using fermented WM for dough preparation produced a reduction of Penicillium expansum *growth of 0.5–0.6 log CFU/g and an improvement in shelf life of 1–2 days in relation to control bread.*

Keywords Fermentation, Whey, Antifungal activity, Shelf life, Lactic acid bacteria.

INTRODUCTION

Whey obtained in the cheese production process is the main waste by-product of the dairy industry. The principal components of whey are lactose, protein, fat, calcium, phosphorus, organic acids and vitamins. Therefore, it is a good culture medium for the growth of bacteria in the laboratory. From a biotechnological point of view, the composition of whey allows the growth of microorganisms, including lactic acid bacteria (LAB), and its fermentation, reducing the biological demand for oxygen and obtaining products with a high added value for the food industry (Karwowska *et al.* 2014; Khem *et al.* 2016). A growing interest in whey has been noted (Figure 1).

During recent years, several studies have reported the beneficial effects of whey. On the one hand, whey protein used to supplement food improves the health of children, adults and the elderly (Stobaugh *et al.* 2016). The peptides generated from the enzymatic hydrolysis of whey proteins show biological effects *in vitro*, such as antimicrobial, antioxidant, antihypertensive and antidiabetic activity among others (Brandelli *et al.* 2015). On the other hand, recent studies evidenced that food containing lactic acid bacteria (LAB) has beneficial effects (Alwan *et al.* 2014; Chen *et al.* 2014). The antifungal activity spectrum of LAB has been very well studied since the food industry showed interest in reducing the use of chemical preservatives to offer 'additive-free' food (Arena *et al.* 2016; Russo *et al.* 2017).

Fungal growth is the most important factor limiting the shelf life of loaf bread, and it is a big problem resulting in significant economic losses. The most dominant fungal species in this type of product are those of the genera *Penicillium* and *Aspergillus* (Marin *et al.* 2003; Garcia *et al.* 2019). Fungal contamination of bakery products usually is after bread processing (Suhr and Nielsen 2003; Smith *et al.* 2004). Although yeast contamination is not common, it can be observed in bakery products stored in a modified atmosphere (Deschuyffeleer *et al.* 2011).

Calcium propionate is used generally to prolong the microbiological shelf life of bread; in Europe, maximum limits of 0.1–0.3% have been established depending on the type of pre-packaged bread (European Union 2011; Belz *et al.*

*Author for correspondence. E-mail: carlos.luz@uv.es

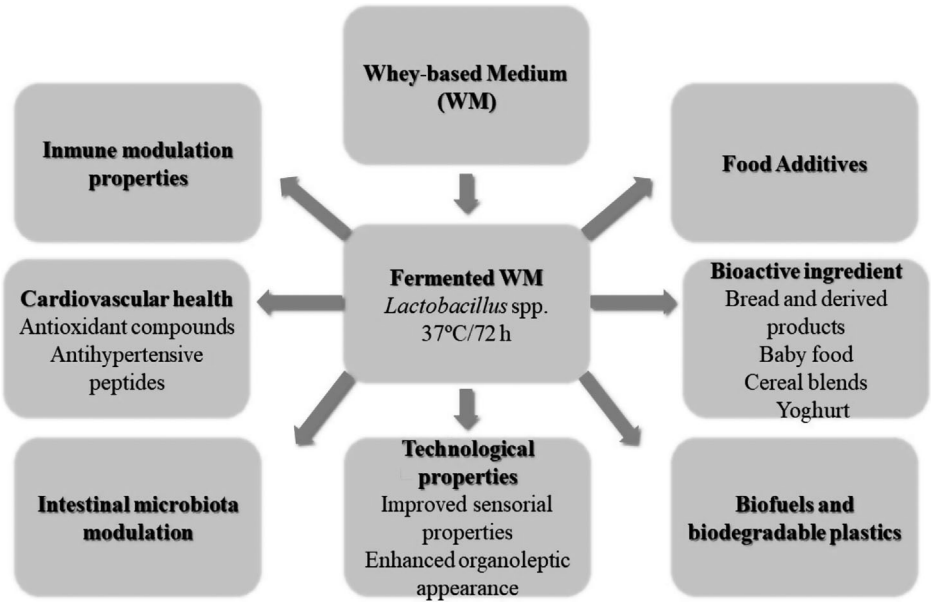

Figure 1 Technological and health-promoting properties of the fermented WM with LAB.

2012). Instead, consumer concern about the negative effects of chemical food preservatives on health is increasing. Therefore, consumers demand of food biopreservation strategies are proliferating (Priftis *et al.* 2007; Shim *et al.* 2011).

The aims of this study were to evaluate (a) the antifungal properties of whey-based medium (WM) fermented by several strains of LAB, (b) characterisation of the antimicrobial compounds produced during WM fermentation through LC-ESI-qTOF mass spectrometry and (c) employment of the WM fermented by *Lactobacillus plantarum* for improving the shelf life of loaf bread inoculated with *Penicillium expansum*.

MATERIALS AND METHODS

Chemicals

Chromatographic solvents acetonitrile (ACN) and ethyl acetate (EA) were obtained from VWR Chemicals (Barcelona, Spain), and formic acid (FA) was obtained from Fisher Scientific (Leicestershire, UK). Dihydroferulic acid, DL-ρ-hydroxyphenyllactic acid, 3,4-dihydroxyhydrocinnamic, 1,2-dihydroxybenzene, benzoic acid, sinapic acid, hydroxycinnamic acid, vanillic acid, hydroxybenzoic acid, ρ-coumaric acid, vanillin, syringic acid, caffeic acid, chlorogenic acid and gallic acid were provided by Sigma-Aldrich (Dublin, Ireland). Ferulic acid was purchased from MP Biomedicals (California, USA), protocatechuic acid from HWI Pharma Services (Ruelzheim, Germany), and DL-3-phenyllactic acid from Bachem (Weil am Rhein, Germany). All analytes had a purity of 95%.

Sodium chloride (NaCl), C18 and anhydrous magnesium sulphate ($MgSO_4$) were provided by Sigma-Aldrich. The culture media potato dextrose broth (PDB), potato dextrose agar (PDA) and De Man Rogosa and Sharpe (MRS) were obtained from Liofilchem (Teramo, Italy). A Milli-Q purification system (Millipore, Bedford, MA, USA) was used to obtain Milli-Q water (<18 MΩ cm resistivity). Gel electrophoresis equipment and molecular weight markers were provided by Bio-Rad (California, USA).

Cow's milk whey powder was obtained from the company Nutriops S.L. (Murcia, Spain) in 440 g pot format. The nutritional composition of the whey without chemical preservative is 71.5 g carbohydrate/100 g, 12.2 g protein/100 g, 0.9 g fat/100 g and 2 g sodium/100 g.

Microbial strains and growth conditions

Strains of *Fusarium verticillioides* CECT 20926, *Fusarium graminearum* CECT 20490, *Fusarium moniliforme* CECT 2982, *Penicillium roqueforti* CECT 2905, *Penicillium camemberti* CECT 2267, *P. expansum* CECT 2278, *Aspergillus parasiticus* CECT 2681 and *Aspergillus niger* CECT 2088 were obtained from CECT (Valencia, Spain). *Aspergillus flavus* ITEM 8111 was obtained from the ITEM microbial culture collection of the Institute of Sciences and Food Production (Bari, Italy). Fungal cryopreservation was carried out in sterile 30% glycerol at –80 °C. At the time of analysis, fungi were cultured first in PDB and later in PDA at 25 °C to obtain spores.

The LAB used in this study were also obtained from CECT: *L. plantarum* CECT 220, *L. plantarum* CECT 221, *L. plantarum* CECT 223, *L. plantarum* CECT 224, *L. plantarum* CECT 748, *L. plantarum* CECT 749 and *L. plantarum* CECT 750. Bacterial cryopreservation was carried out in sterile 30% glycerol at −80 °C.

After the recovery period, in MRS broth at 37 °C for 48 h under anaerobic conditions, bacteria were inoculated at a concentration of 10^8 CFU/mL in WM and incubated for 24, 48 and 72 h at 37 °C. Whey-based medium was constituted by 10% whey powder and 90% sterile water. After the incubation period, fermented WM was centrifuged in an Eppendorf 5810R centrifuge (Hamburg, Germany) at 4000 rpm for 10 min at 4 °C. The cell-free supernatant (CFS) of fermented WM was dried using a FreeZone 2.5 Liter Benchtop Freeze Dryer (Labconco, Missouri, USA) and stored at −19 °C.

Molecular mass estimation

The degree of protein hydrolysis of WM fermented with LAB for 24, 48 and 72 h was analysed by SDS-PAGE using a 15% (w/v) separating gel and 4% stacking gel (El-Ghaish *et al.* 2010). For visualisation of protein bands, gels were stained in a staining solution: 0.1% Brilliant Blue R-250, 50% water, 40% methanol and 10% acetic acid. Molecular mass of proteins was evaluated by comparison with the Bio-Rad protein ladder standards Precision Plus Protein (high MW range) and Natural Polypeptide SDS-PAGE (low MW range).

Determination of phenolic acids by HPLC-ESI-Q-TOF-MS

The fermented WM was purified using the QuEChERS method before phenolic acid analysis (Brosnan *et al.* 2014). The purification method consisted of two steps. In the first, 10 mL of fermented WM was vortexed for 1 min and then extracted with 10 mL EA (1% FA) and salts (4 g of $MgSO_4$ and 1 g of NaCl). In the second step, after centrifugation, 150 mg C18 and 900 mg $MgSO_4$ were added to the supernatant and the mixture was vortexed for 1 min. Finally, samples were centrifuged again, and the supernatants were evaporated under nitrogen flow. Before chromatographic analysis, samples were resuspended in 1 mL of 10% ACN and filtered with a 0.22-μm pore size filter.

Chromatographic analyses were performed by an Agilent 1200 (California, USA), which consisted of a autosampler, vacuum degasser and binary pump. A Gemini C18 column (50 × 2 mm, 100 Å and particle size 3 μm; Phenomenex) was used for chromatographic separations. The mobile phase consisted of 0.1% FA in water (solvent A) and 0.1% FA in ACN (solvent B). For the analysis of phenolic acids, the optimised elution gradient was the following: 0 min, 5% B; 30 min, 95% B; 35 min, 5% B. The system was re-equilibrated for 3 min; the total run-time was 37 min. Sample elution was at flow rate of 0.3 mL/min, and the sample volume injected was 20 μL.

Mass spectrometry analyses were performed by using a Q-TOF-MS (6540 Agilent Ultra High Definition Accurate Mass) coupled to an Agilent Dual Jet Stream electrospray ionisation (Dual AJS ESI) interface operating in the negative ion mode. The following parameters were as follows: fragmentor voltage was 175 V; capillary voltage 3.5 kV; nebuliser pressure 30 psig; and drying gas flow (N_2) 8.0 L/min and temperature 350 °C. Targeted MS/MS experiments were carried out using collision energy of 10, 20 and 40 eV. Integration and data elaboration were managed using MassHunter Qualitative Analysis Software B.08.00 (Denardi-Souza *et al.* 2018).

Determination of antifungal activity by agar diffusion method

100 μL of fermented WM for 72 h suspended in PDB at a concentration of 250 mg/mL was added to PDA plates contaminated with fungal spores. Plates were incubated at 25 °C for 3 days (Madhyastha *et al.* 1994). After that, we studied growth inhibition by measuring the diameter of inhibition halos, considering as positive only halos larger than 8 mm (Castlebury *et al.* 1999).

Determination of antifungal activity by microdilution method

100 μL of fermented WM for 72 h suspended in PDB at final concentration from 0.5 to 250 mg/mL was added to sterile microplates (96 well). After, all wells except negative control (noncontaminated PDB medium) were contaminated with 100 μL of a 5×10^4 spores/mL suspension of the toxigenic fungi. The positive control consisted of contaminated medium with nonfermented WM (250 mg/mL). After that, microplates were incubated at 25 °C for 72 h. The minimum inhibitory concentration (MIC) was considered as the concentration of fermented WM for which no visible fungal growth was observed on wells (Fothergill 2012).

After determining the MIC, concentrations equal to and higher than the MIC were cultivated on PDA plates to determine the minimum fungicidal concentration (MFC). After incubation (3 days at 25 °C), the MFC was the concentration at which no visible fungal growth was evidenced. Four replicates of each assay were carried out.

Baking and bread treatment

The loaf bread samples were elaborated by following the recipe: 300 g of wheat flour, 175 g of tap water, 20 g of yeast for bakery products (Levital, Spain), 10 g of sucrose and 5 g of NaCl. Doughs were prepared by replacing 50% (WM50) and 100% (WM100) of the total water in the bread with nonlyophilised WM fermented for 72 h by the selected strain. To compare the inhibition of fungal growth, we also made a control bread without additives and a commercial control bread with 0.2% calcium propionate.

After that, the doughs were homogenised in a bakery machine (Silver Crest) for 15 min and fermented for 1 h at 25 °C. In total, four doughs of bread samples for each test were placed on a perforated greased plate and baked at 200 °C for 40 min in a Memmert ULE 500 muffle furnace

Table 1 Antifungal activity on solid medium of PDA of whey protein fermented with 7 strains of lactic acid bacteria and against several toxigenic fungi.

	Lactic acid bacteria																	
	Lactobacillus plantarum *220*			Lactobacillus plantarum *221*			Lactobacillus plantarum *223*			Lactobacillus plantarum *224*			Lactobacillus plantarum *748*			Lactobacillus plantarum *749*			Lactobacillus plantarum *750*		
Fungi	*24 h*	*48 h*	*72 h*	*24 h*	*48 h*	*72 h*	*24 h*	*48 h*	*72 h*	*24 h*	*48 h*	*72 h*	*24 h*	*48 h*	*72 h*	*24 h*	*48 h*	*72 h*	*24 h*	*48 h*	*72 h*
Penicillium camemberti	−	−	−	+	+	+	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−
Penicillium expansum	−	−	−	−	+	+	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−
Penicillium roqueforti	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−
Aspergillus parasiticus	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−
Aspergillus flavus	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−
Aspergillus niger	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−
Fusarium moniliformis	−	+	+	−	+	+	+	+	+	−	+	+	−	+	+	+	+	+	−	+	+
Fusarium verticillioides	+	+	+	+	+	+	+	+	+	−	+	+	−	+	+	+	+	+	−	+	+
Fusarium graminearum	+	+	+	+	+	+	−	+	+	+	+	+	+	+	+	−	+	+	−	−	−

(Madrid, Spain). After baking, bread samples were cooled to room temperature (20–22 °C, 1 h approximately). Then, breads were cut into 30 g slices and immediately prepared to be inoculated with *P. expansum* fungi. Nine replicates for each typology of bread were inoculated in nine spots with 100 μL of a suspension containing 3×10^5 spores/mL. Finally, they were packed in sealed low-density polyethylene bags with the help of a Sammic TS-150 thermosealer (Basarte, Spain). All plastic bags were closed hermetically and incubated at room temperature for 7 days. During the preservation period, visual control of the surface of the inoculated bread was carried out daily, identifying at a glance the possible growth or not of fungus and to establish the effect of the treatment on the shelf life (Dal Bello *et al.* 2007).

Antimicrobial activity: determination of the fungal load of bread

Once the period of preservation of the bread samples was exceeded, a microbiological study was carried out. After 7 days of incubation, 40 g of bread was homogenised with 360 mL of 0.1% peptone water, previously autoclaved, in an IUL Stomacher (Barcelona, Spain) for 30 s. From that, three serial decimal dilutions were prepared in glass tubes with 9 mL of peptone water. Subsequently, 100 μL from each tube was plated out in PDA culture medium plates. The plates were incubated at 26 °C, and the number of viable colonies was counted after 72 h of incubation (Torrijos *et al.* 2019).

RESULTS AND DISCUSSION

Identification of phenolic compounds and protein fraction obtained by WM powder fermented by several strains of *L. plantarum*

The phenolic acids determined in the fermented WM are shown in Table 1. In particular, it is possible to show in the results that the strain of *L. plantarum* that produced the lowest amount of phenolic acids was strain CECT 220, considering that just four antimicrobial compounds were identified. Considering the compounds detected, we have to underline the importance from a microbiological point of view of hydroxybenzoic acid and DL-3-phenyllactic acid (Barman *et al.* 2017; Taofiq *et al.* 2017).

In particular, Barman *et al.* (2017) studied growth reduction of *Mucor* sp., *Penicillium digitatum* and *Trichophyton rubrum* by using the CFS of *L. plantarum* at a concentration of 10 mg/mL. In addition, they evidenced the presence of lactic acid, phenyllactic acid and other compounds unidentified in CFS. This experiment showed the high potential of the strain *L. plantarum* to improve the shelf life of bread contaminated by *Bacillus subtilis* and *Mucor* sp.

The strain of *L. plantarum* that showed the highest capacity to produce phenolic compounds (nine compounds,

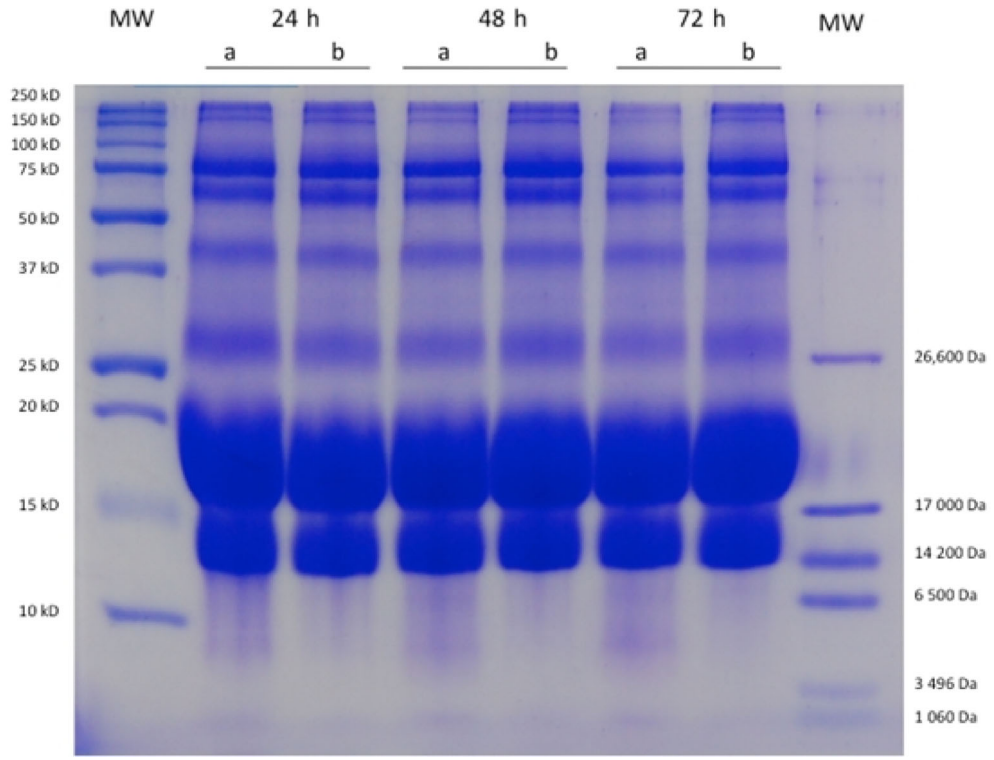

Figure 2 SDS-PAGE separation of WM proteins fermented by (a) *Lactobacillus plantarum* CECT 221 and (b) *L. plantarum* CECT 750 at 24, 48 and 72 h of incubation.

including several antimicrobial molecules like chlorogenic acid, syringic acid, benzoic acid and DL-3-phenyllactic acid) was strain CECT 221. The other strains of *L. plantarum* produced between four and six antimicrobial phenolic compounds.

Figure 2 shows the SDS-PAGE separation gel for the WM powder protein fraction after fermentation of the matrix with different strains of *L. plantarum* for 24, 48 and 72 h. In particular, it is possible to observe a decrease in the band intensity of the proteins with a molecular weight ranging from 100 to 250 kDa, and the decrease in the protein fraction observed is proportional to the incubation time. More assays have to be performed to understand the possible peptide fraction that could be responsible also for the antifungal activity.

Antifungal activity and MIC/MFC of WM fermented with LAB

Table 2 shows the data related to the antimicrobial activity of the WM fermented by several bacteria on nine different toxigenic fungi of the genera *Penicillium*, *Aspergillus* and *Fusarium*, using antimicrobial tests on solid PDA medium. The fermented WM produced through a biotechnological fermentation process using *L. plantarum* strain CECT 221 presented antifungal activity against *P. camemberti* and *P. expansum* and also on the three *Fusarium* strains tested, whereas the ingredient tested did not show any antimicrobial effect on the strains of *P. roqueforti* and *Aspergillus* used. The WM fermented by *L. plantarum* CECT 220, 223, 224, 748, 749 and 750 all showed the same antifungal effect, in particular on the three *Fusarium* strains used in this study.

The MIC and MFC of the WM fermented by different strains of *Lactobacillus* spp. for 72 h are summarised in Table 3. In particular, for the *Penicillium* strains, the MIC and MFC of the WM ranged from 15.6 to 250 mg/mL. The highest antimicrobial activity was evidenced by the strains *L. plantarum* CECT 221, 749 and 750, with an MFC of 62.5 mg/mL, whereas the lowest activity was evidenced by the strains *L. plantarum* CECT 220, 223 and 224, for which the MFC detected was 250 mg/mL. Analysing the results related to the antimicrobial activity of the WM fermented by LAB on *Aspergillus* strains, it is possible to observe that the activity presented by the fermented matrices is lower than for the *Penicillium* strains, due probably to some resistance factors typical of the genus *Aspergillus* against many antimicrobial compounds. In particular, for *A. parasiticus*, the MIC and MFC for all the fermented WM were 250 mg/mL, and these data were also confirmed on the strain of *A. flavus*. The fermentation matrices produced by *L. plantarum* CECT 748 and 749 did not show any antifungal activity on the toxigenic strain tested. On the strain *A. niger*,

Table 2 MIC-MFC expressed in mg/mL evidenced by the extract obtained by the whey protein fermented with 7 strains of lactic acid bacteria and against several toxigenic fungi.

	Penicillium camemberti		Penicillium expansum		Penicillium roqueforti		Aspergillus parasiticus		Aspergillus flavus		Aspergillus niger		Fusarium moniliformis		Fusarium verticillioides		Fusarium graminearum	
Lactic acid bacteria	*MIC*	*MFC*	*MIC*	*MFC*	*MIC*	*MFC*	*MIC*	*MFC*	*MIC*	*MFC*	*MIC*	*MFC*	*MIC*	*MFC*	*MIC*	*MFC*	*MIC*	*MFC*
Lactobacillus plantarum CECT 220	62.5	250	62.5	250	125	125	250	250	250	250	250	250	62.5	125	62.5	62.5	31.3	250
Lactobacillus plantarum CECT 221	125	125	15.6	62.5	125	125	250	250	250	250	nd	nd	125	250	125	125	62.5	250
Lactobacillus plantarum CECT 223	62.5	250	125	250	nd	nd	250	250	250	250	nd	nd	250	250	125	125	62.5	250
Lactobacillus plantarum CECT 224	62.5	250	62.5	250	nd	nd	250	250	250	250	nd	nd	62.5	250	62.5	125	62.5	250
Lactobacillus plantarum CECT 748	nd	nd	250	nd	125	125	250	250	nd	nd	nd	nd	250	250	250	250	125	125
Lactobacillus plantarum CECT 749	125	125	125	250	125	125	250	250	nd	nd	nd	nd	125	250	125	125	62.5	62.5
Lactobacillus plantarum CECT 750	125	125	62.5	250	125	125	250	250	250	250	nd	nd	125	250	125	125	62.5	62.5

only the fermentation matrix produced by *L. plantarum* CECT 220 showed MIC and MFC at 250 mg/mL, whereas the other fermentation matrices produced by the other strains of LAB did not show any antifungal effect on these toxigenic fungi.

Related to the antimicrobial activity of the WM fermented by LAB on the *Fusarium* strains, the fermented matrices presented an antifungal effect similar to the activity shown for *P. camemberti* and *P. expansum*; in particular, the MIC and MFC ranged from 31.3 to 250 mg/mL. The most active fermented matrices were produced by *L. plantarum* CECT 749 and 750 which had MIC and MFC of 62.5 mg/mL for the strain *F. graminearum*. The WM fermented by *L. plantarum* CECT 748 showed the lowest antifungal activity, in particular on the strains *Fusarium moniliformis* and *F. verticillioides*, for which the MIC and MFC were 250 mg/mL, respectively.

Gamba *et al.* (2016) reported that whey obtained from the fermentation of milk by the kefir grains CIDCA AGK1 showed antifungal activity against *F. graminearum*, and the production of zearalenone was not detected in the treatment of higher concentrations of whey.

Shelf life improvement of bread treated with fermented WM

Whey is considered the main residue of the cheese and dairy industry. It has a high nutritional value and can be used to obtain valued products such as lactic acid, biodegradable polymers and bioethanol, among others. In the first case, the fermentative processes of whey carried out by bacteria and yeasts have been directed to obtain compounds of interest and to produce new ingredients and/or foods (Pescuma *et al.* 2015).

Related to the results for the shelf life of the breads treated with 0.2% calcium propionate (E282) and WM fermented with *L. plantarum* CECT 221 as a replacement for the water used for bread dough preparation, the data are shown in Table 4. On the one hand, the control bread without any additives showed visible fungal growth after 3 days. Nevertheless, the commercial control bread with additive E282 and WM50 bread evidenced an increment in shelf life of 1 day compared to the control bread (Figure 3). On the other hand, the WM100 bread showed fungal growth at 5 days of incubation. Therefore, the replacement of all water by fermented WM provided an increment in shelf life of 1 and 2 days, respectively, in comparison with the commercial control bread and WM50 bread.

The data shown for the visible shelf life of the bread loaves treated with WM fermented by *L. plantarum* and contaminated with *P. expansum* were also confirmed by microbiological analysis of the population of fungal contaminants in the food matrix studied. In particular, the control bread at 7 days of incubation presented a fungal population of 5.1 log CFU/g, whereas for the commercial control

Table 3 Phenolic compounds identified in lyophilised crude extract, through LC-qTOF-MS, obtained from WM fermented by seven strains of *Lactobacillus plantarum.*

Compound	*Molecular formula*	*MW*	Lactobacillus plantarum *220*	Lactobacillus plantarum *221*	Lactobacillus plantarum *223*	Lactobacillus plantarum *224*	Lactobacillus plantarum *748*	Lactobacillus plantarum *749*	Lactobacillus plantarum *750*
Gallic	$C_7H_6O_5$	170,12	−	−	−	−	−	−	−
Protocatechuic	$C_7H_6O_4$	154,12	−	−	−	−	−	−	−
Chlorogenic	$C_{16}H_{18}O_9$	354,31	−	+	−	−	−	−	−
Caffeic	$C_9H_8O_4$	180,16	−	−	−	−	+	+	−
Syringic	$C_9H_{10}O_5$	198,17	−	+	−	−	−	+	+
Vanillin	$C_8H_8O_3$	152,13	−	−	−	−	−	+	+
p-Coumaric	$C_9H_8O_3$	164,16	−	−	−	−	+	+	+
Ferulic	$C_{10}H_{10}O_4$	194,18	−	+	+	+	−	−	+
Hydroxybenzoic	$C_7H_6O_3$	138,12	+	+	+	−	−	−	−
Vanillic	$C_8H_8O_4$	168,15	−	−	−	−	−	−	+
Salicylic	$C_7H_6O_3$	138,12	−	+	+	+	+	−	−
Hydrocinnamic	C_9H10O_2	150,17	−	−	−	−	+	−	−
Sinapic	$C_{11}H_{12}O_5$	224,21	+	+	+	+	−	−	+
Benzoic	$C_7H_6O_2$	122,12	+	+	+	+	−	−	−
DL-3-phenyllactic	$C_9H_{10}O_3$	166,17	+	+	+	−	+	−	−
Dihydroferulic	$C_{10}H_{12}O_4$	196,2	−	−	−	−	−	−	−
1-2 Dihydroxybenzene	$C_6H_60_2$	110,11	−	−	−	−	+	−	−
3,4-Dihydroxyhydrocinnamic	$C_9H_{10}O_4$	182,17	−	−	−	−	−	−	−
DL-p-Hydroxyphenyllactic	$C_9H_{10}O_4$	182,17	−	+	−	−	−	−	−
3-(4-Hydroxyphenyl) propionic	$C_9H_{10}O_3$	166,17	−	−	−	−	−	−	−

Table 4 Shelf life monitored in days, of the bread loaves contaminated with *Penicillium expansum* and treated with whey calcium propionate at 0.2% (E282), WM fermented with *Lactobacillus plantarum* CECT 221 in replacing of the 50 and 100%, respectively, of the water used for the bread dough preparation.

	Days						
Treatments	*1*	*2*	*3*	*4*	*5*	*6*	*7*
Control	–	–	+	+	+	+	+
E282	–	–	–	+	+	+	+
WM50%	–	–	–	+	+	+	+
WM100%	–	–	–	–	+	+	+

The treatments were also compared with a control test consisting of bread loaves contaminated with *Penicillium expansum* without any preservative ingredient.

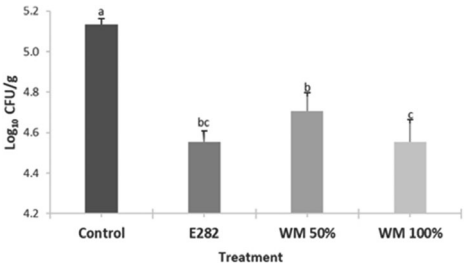

Figure 4 Population of the *Penicillium expansum* in bread loaves treated with calcium propionate at 0.2% (E282), WM fermented with *Lactobacillus plantarum* CECT 221 in replacing of the 50 and 100%, respectively, of the water used for the bread dough preparation, in comparison with the control bread.

bread, it was 4.6 log CFU/g, the difference of 0.5 log CFU/g in comparison with the control bread being statistically significant. The treatment that produced the smallest fungal charge at 7 days of incubation was WM100, for which the *P. expansum* population detected was 4.5 log CFU/g, a statistically significant fungal reduction of 0.2 and 0.6 log CFU/g in comparison with the WM50 bread and control bread, respectively. No significant differences were observed with respect to bread with additive (Figure 4).

Microbial fermentation is one of the oldest and most economically and ecologically friendly methods of preserving foods (Zannini *et al.* 2012). In this context, the use of chemical compounds is not recommended, and natural solutions are frequently proposed for food preservation. Nowadays, consumers are looking for natural products and less processed and clearly safer products. In this context, the possibility of obtaining antifungal compounds, deriving from a fermentation process, capable of reducing the percentage of fungal growth represents a growing interest as an alternative to chemical preservation. Several reviews have examined the antifungal activity spectrum of LAB and production of secondary metabolites, as well as their interactions with mycotoxins (Dalie *et al.* 2010; Crowley *et al.* 2013).

Dal Bello *et al.* (2007) showed improvement in the shelf life of breads using the antifungal strain *L. plantarum* FST1.7. The production of lactic acid, phenyllactic acid and two cyclic dipeptides (L-Leu-L-Pro and L-Phe-L-Pro) in the culture media and sourdough were directly related to the antifungal activity (Ryan *et al.* 2009a, 2009b).

CONCLUSION

Whey powder fermentation using different strains of *L. plantarum* produced several phenolic acids and possible peptides; their maximum antimicrobial activity in bread

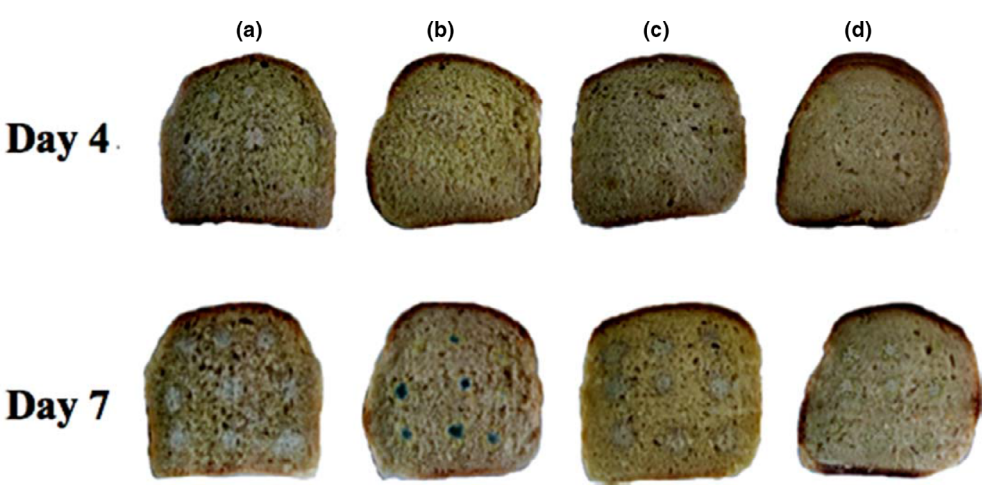

Figure 3 Bread loaves contaminated with *Penicillium expansum* (a) control and treated with (b) 0.2% of calcium propionate (c) and (d) WM fermented with *Lactobacillus plantarum* CECT 221 in replacing of the 50 and 100%, respectively, of the water used for the bread dough preparation, at 4 and 7 days of incubation.

against *P. expansum* was shown on the fifth day of treatment, with an increment in shelf life of the bread treated.

The whey powder fermented by strains of *L. plantarum* demonstrated a high antifungal activity against several toxigenic fungi of the genera *Aspergillus*, *Fusarium* and *Penicillium* on solid and liquid media. This study demonstrates the importance of whey as a culture medium for *L. plantarum* and the production of bioactive metabolites with antimicrobial activity. Further studies will be focused on identification and characterisation of the peptide fraction produced during whey fermentation and its possible application as a food ingredient and for nutraceutical purposes.

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