



Lactobacillus plantarum with broad antifungal activity: A promising approach to increase safety and shelf-life of cereal-based products

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ABSTRACT

Cereal-based fermented products are worldwide diffused staple food resources and cereal-based beverages represent a promising innovative field in the food market. Contamination and development of spoilage filamentous fungi can result in loss of cereal-based food products and it is a critical safety concern due to their potential ability to produce mycotoxins. Lactic Acid Bacteria (LAB) have been proposed as green strategy for the control of the moulds in the food industry due to their ability to produce antifungal metabolites. In this work, eighty-eight *Lactobacillus plantarum* strains were screened for their antifungal activity against *Aspergillus niger*, *Aspergillus flavus*, *Fusarium culmorum*, *Penicillium roqueforti*, *Penicillium expansum*, *Penicillium chrysogenum*, and *Cladosporium* spp. The overlaid method was used for a preliminary discrimination of the strains as no, mild and strong inhibitors. *L. plantarum* isolates that displayed broad antifungal spectrum activity were further screened based on the antifungal properties of their cell-free supernatant (CFS). CFSs from *L. plantarum* UFG 108 and *L. plantarum* UFG 121, in reason of their antifungal potential, were characterized and analyzed by HPLC. Results indicated that lactic acid was produced at high concentration during the growth phase, suggesting that this metabolic aptitude, associated with the low pH, contributed to explain the highlighted antifungal phenotype. Production of phenyllactic acid was also observed. Finally, a new oat-based beverage was obtained by fermentation with the strongest antifungal strain *L. plantarum* UFG 121. This product was submitted or not to a thermal stabilization and artificially contaminated with *F. culmorum*. Samples containing *L. plantarum* UFG 121 showed the best biopreservative effects, since that no differences were observed in terms of some qualitative features between not or contaminated samples with *F. culmorum*. Here we demonstrate, for the first time, the suitability of LAB strains for the fermentation and antifungal biopreservation of oat-based products.

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1. Introduction

Cereal-based fermented products are worldwide food resources essential for human nutrition, since they are an important source of protein, dietary fibers, carbohydrates, as well as micronutrients (Guyot, 2012). In the last years, functional fermented non-dairy foods/beverages from cereal origin are globally gaining in interest (Leroy and De Vuyst, 2014) in reason of the versatility in answering to the major trends in food consumption (vegetarian, vegan, low-fat, low-salt). However, contamination with spoilage filamentous fungi remains a major threat for the cereal-based market. Losses of food due to fungal spoilage are difficult to estimate (Pitt and Hocking, 2009). Moreover, contamination by moulds poses a critical safety concerns due to their potential ability to

produce mycotoxins (Oliveira et al., 2014). The most common contaminant moulds responsible for spoilage of cereal matrices belong to the genera *Penicillium*, *Fusarium*, *Aspergillus*, *Cladosporium*, and *Rhizopus*. In general, the control of filamentous fungi in the food industry relies on physical and chemical treatments. In the last years, eco-friendly preservatives approaches have been emerging, including the employment of lactic acid bacteria (LAB) with antifungal properties. Due to their large safe history of use for food fermentations, LAB can be deliberately added into the food chain, suggesting that they could be integrated into food systems as natural food preservatives (Oliveira et al., 2014; Pawlowska et al., 2012).

LAB antimicrobial activity has been attributed to a wide variety of active antagonistic metabolites mainly including: organic acids, carbon dioxide, ethanol, hydrogen peroxide, fatty acids, acetoin, diacetyl, cyclic dipeptides, bacteriocins, or bacteriocin-like inhibitory substances (reviewed by Crowley et al., 2013a). Lactic and acetic acids are the main products of the fermentation of carbohydrates by LAB and they could exert antifungal activity (Dalie et al., 2010). Although still unclear, the inhibition mechanism of weak organic acids seems to involve

Abbreviations: CFS, cell-free supernatant; LAB, lactic acid bacteria; PLA, phenyllactic acid.

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cytoplasm acidification and failure of proton motive forces, probably due to the different solubility within the plasma membrane, between the dissociated and non-dissociated forms of such molecules (Reis et al., 2012). Among organic acids, phenyllactic acid (PLA) displays a broad spectrum of antifungal activity (Lavermicocca et al., 2000; Rizzello et al., 2011; Ström et al., 2002). However, it was hypothesized that synergistic mechanism between organic acids and other metabolites could substantially increase the overall antimicrobial activity (Cortés-Zavaleta et al., 2014). For this reason, growing efforts are addressed to discover new antifungal molecules, their interactions, and the molecular basis that rules the corresponding metabolic pathways. Nowadays, new antifungal metabolites can be purified and characterized due to the availability of complex analytical approaches (Brosnan et al., 2012). Moreover, advances in genomic and physiology of LAB allow to link individual genetic and metabolic traits of starter cultures to specific food quality attributes (Gänzle, 2009).

Several studies have aimed to identify by large scale *in vitro* screening LAB strains with interesting antifungal potential (Cheong et al., 2014; Crowley et al., 2012a; Magnusson et al., 2003). As a consequence, LAB strains showing broad spectrum of antifungal activity have been proposed for application into different model food system including bread and sourdough (Axel et al., 2015; Dal Bello et al., 2007), malting process (Oliveira et al., 2015), dairy products (Cheong et al., 2014; Voulgari et al., 2010), fruit and vegetables (Crowley et al., 2012a).

In the present study, eighty-eight *Lactobacillus plantarum* strains were screened for their ability to inhibit seven fungal species that are common contaminant of cereal matrices. The cell-free supernatants from strains showing a broad antagonistic spectrum were analyzed by HPLC in order to quantify the main organic acids produced. A technological application was investigated by using as *in situ* food model system a new oat-based product which was fermented by the *L. plantarum* strain with the strongest antifungal activity and artificially contaminated with *Fusarium culmorum*.

2. Materials and methods

2.1. Bacterial strains and growth conditions

Eighty-eight *L. plantarum* strains available at the Laboratory of Industrial and Molecular Microbiology of the University of Foggia (Foggia, Italy) were previously isolated from different food matrices and stored at -80°C in MRS broth (Oxoid, Basingstoke, UK) supplemented with 20% of glycerol. For the experiments, *L. plantarum* strains were routinely grown in MRS broth and incubated at 30°C for 24 h.

2.2. Fungal strains and growth conditions

Six filamentous fungi, namely *Aspergillus niger* CECT 2805, *Aspergillus flavus* CECT 20802, *Penicillium roqueforti* CECT 20508, *Penicillium expansum* CECT 2278, *Penicillium chrysogenum* CECT 2669, and *F. culmorum* CECT 2148 were acquired by the Spanish Type Culture Collection (CECT, Paterna, Spain). A strain identified as *Cladosporium* spp. UFG 163 was previously isolated from an oat based matrix. Lyophilized cultures of moulds were resuspended on malt extract broth (Oxoid) and incubated at 24°C for 24 h. Then, moulds cultures were plated on malt extract agar and incubated at 24°C for further 5 days. Fungal spore suspensions were prepared by brushing the plate surface with Buffered Peptone Water (BPW) (Oxoid) using a sterile swab. Then, spores were resuspended in BPW containing 0.1 % Tween 80, and stored at 4°C until further use.

2.3. Screening of the antifungal activity of *L. plantarum* strains

The overlaid method was used for a preliminary screening of the antifungal activity of the all *L. plantarum* strains according to Cheong et al. (2014). Briefly, 5 μL of *L. plantarum* cultures at middle exponential

phase were spotted on MRS agar plates. After 24 h of incubation at 30°C , the plates were overlaid with 10 mL of soft malt extract agar (0.75% agar) supplemented with 100 μL of malt extract broth containing approximately 1×10^6 (Chtioui et al., 2014) spores mL^{-1} of each mould ($\text{OD}_{600} = 0.5$). After 5 days of incubation at 24°C , *L. plantarum* strains were discriminated on the basis of the halo of inhibition surrounding the spots and were classified as strains of no (\pm), mild (+), or strong (++) inhibition showing inhibition zone lower than 1 mm, ranging from 1 to 3 mm, or more than 3 mm, respectively.

2.4. Antifungal activity of *L. plantarum* cell-free supernatants

L. plantarum strains that displayed very strong antifungal activity were grown on MRS broth for 16–18 h at 30°C until early stationary phase ($\text{OD}_{600} = 1.6$), corresponding to approximately 2×10^9 CFU mL^{-1} according to previously generated standard growth curves. The corresponding supernatant was recovered by centrifugation ($8000g \times 5$ min), and sterilized by filtration (0.2 μm filters, VWR international, West Chester, PA). Plates of malt extract agar were supplemented with sterile cell-free supernatant (CFS) at increasing concentration (namely, 0%, 4%, 8%, 12%, v/v). Petri dishes used as control were supplemented with the same amounts of sterile MRS broth. 10 μL of a culture of each mould containing approximately 1×10^6 (Chtioui et al., 2014) spores mL^{-1} were spotted at the center of the plates. The antifungal ability was determined by hyphal radial growth inhibition after 3 and 5 days of incubation at 24°C in presence of increasing concentrations of microbial CFS. Experiments were performed in triplicate.

2.5. Characterization of antifungal compounds

The antagonistic activity and the nature of the inhibitory substances secreted by each *L. plantarum* strain were investigated by well diffusion assays as described by Al Kassaa et al. (2014). The cell free supernatant was obtained from each culture grown in MRS broth for 18 h at 30°C . Briefly, bacteria cultures were centrifuged ($8000 \times g$ for 20 min, 4°C) and supernatants were sterilized by filtration through Millex-GV 0.22 μm hydrophilic Durapore PVDF membrane (Millipore, Billerica, MA, USA). Then, each filtered CFS (CFS-A) was exposed to temperature of 80°C for 10 min (CFS-B) and neutralization with 2 M NaOH (pH 6.5) (CFS-C). Successively, each neutralized supernatant (CFS-C) was subject to enzymatic and heat treatment as it follows: incubation with catalase (1 mg mL^{-1}) (Sigma-Aldrich Corporation, USA) at 37°C for 1 h (CFS-D); incubation with of proteinase K (1 mg mL^{-1}), trypsin (1 mg mL^{-1}), α -chemotrypsin (1 mg mL^{-1}), and papain (1 mg mL^{-1}) (all from Sigma) (CFS-E, CFS-F, CFS-G, and CFS-H respectively), incubation at 80°C for 60 and 90 min (CFS-I and CFS-L), 100°C for 60 and 90 min (CFS-M and CFS-N), and at 121°C for 15 min (CFS-O). Wells (6 mm of diameter) were made on MRS agar plates, filled with 100 μL of each CFS and the plates were incubated for 2 h at 4°C in order to permit the diffusion of CFSs into MRS agar. Overnight cultures of fungi were inoculated (1% v/v) into fresh malt extract soft agar (0.75% agar, w/v) and poured over MRS agar plate containing CFSs. All plates were incubated at 37°C for 24 h and, then, the inhibition zones around the wells were measured. Assays were carried out in triplicate.

2.6. Quantification of lactic acid and phenyllactic acid in CFS

To quantify the production of lactic acid in the CFS collected from *L. plantarum* cultures, samples were analyzed by chromatographic method. Each strain (1% v/v, initial cell concentration) was inoculated in MRS media at 30°C , aliquots of culture media (1 mL) were collected at 0, 6, 7, 8 and 24 h and CFS was obtained by centrifugation for 20 min at $8000 \times g$ followed by filtration through a 0.22 μm Millipore filter (Al Kassaa et al., 2014). Then, 20 μL of each CFS were injected into an HPLC

Spectra System P1000XR (Thermo Electron Corporation, Madison, WI, USA), coupled to refractometer Spectra SYSTEM RI-150 (Thermo Electron Corporation), using a Fast Fruit Juice column (50 Å 7 µm, 150 × 7.8 mm, Waters), H₃PO₄ (0.05%) as mobile phase at a flow rate of 0.8 mL min⁻¹ (Chtioui et al., 2014).

2.7. Antifungal activity in fermented oat-based products

Finely milled whole grain oat flour was provided by Glucanova (Lund, Sweden). Concisely, oat flour (18% w/v) and distilled water (82% v/v) were mixed to obtain a final volume of 100 mL. The mixture was heated at 95 °C in a water bath for 10 min, with manual shaking every 2 min, according to previously reported (Coda et al., 2012; Russo et al., 2016). To start the fermentation, cells from *L. plantarum* starter culture at late exponential phase were harvested by centrifugation (5000g × 10 min), washed twice in sterilized saline solution (8.6% NaCl), and inoculated into the food matrix to obtain a concentration of approximately 8 × 10(Coda et al., 2012) cfu g⁻¹. Fermentation was carried out at 30 °C for 16 h with orbital shaking (150 rpm) (Russo et al., 2016). At this time, samples containing live microorganisms were stored at 4 °C for 21 days, while pasteurized samples were submitted to a thermal treatment of 30 min at 65 °C before storage. Pasteurized and unpasteurized samples were artificially inoculated with *F. culmorum* (about 1 × 10(Chtioui et al., 2014) spores mL⁻¹). Control samples were contaminated with *F. culmorum* without a previous fermentation with *L. plantarum* UFG 121. Each experimental condition was repeated in duplicate. The LAB microbial viability was checked by plate counting after 7, 14, and 21 days of storage at 4 °C. At the same experimental times, images of fermented oat-based samples were acquired by using a computer vision system equipped with a digital color camera (EOS 00D, Canon, Melville, NY, USA) located vertically over the matte black background at a distance of 0.45 m (Baiano et al., 2015). A panel of five people carried out a qualitative evaluation of the fermented oat-based samples at each experimental time. The samples were coded with a random 3-digit number in order to mask the treatment identity and to minimize subjectivity. The sensorial attributes judged during evaluation were: overall appearance, color, firmness, odor, off odor, visual quality, occurrence of moulds. Every attribute was scored on a 1 to 5 scale, where 1 = typical/highest perception of quality; 5 = atypical/lowest perception of quality.

3. Results

3.1. Screening of *L. plantarum* strains with antifungal activity

In this work, eighty-eight *L. plantarum* strains, previously isolated from different food matrices, were screened for their antifungal activity against seven moulds belonging to the species *A. niger*, *A. flavus*, *F. culmorum*, *P. roqueforti*, *P. expansum*, *P. chrysogenum*, and *Cladosporium* spp. These moulds were chosen because representative of frequent spoilages of a wide range of food matrices and/or for their potential to produce mycotoxins. The overlaid method was used for a fast initial screening according to Cheong et al. (2014). On the basis of the halo of inhibition around the spots, *L. plantarum* isolates were classified into three groups with no, mild, or strong antifungal ability, respectively. *P. roqueforti* CECT 20508, *A. niger* CECT 2805, *A. flavus* CECT 20802, and *Cladosporium* spp. UFG 163 were the most resistant fungal strains, since between 60% and 80% of the tested *L. plantarum* strains were unable to inhibit their growth (Fig. 1). In contrast, most of the *L. plantarum* analyzed (about 75%) exerted a strong inhibition against *P. chrysogenum* CECT 2669. *P. expansum* CECT 2278, and *F. culmorum* CECT 2148 were strongly inhibited by approximately a 45% of the *L. plantarum* isolates (Fig. 1). However, only few *L. plantarum* strains exhibited a wide spectrum of strong antifungal activity (data not shown). Therefore, nine *L. plantarum* isolates were selected for further investigations, since they were able to strongly inhibit at least three moulds and they were ineffective against no more than three filamentous fungi (Table 1).

3.2. Antifungal activity of CFS

The antifungal activity of the CFSs from the strongest inhibitor *L. plantarum* strains was preliminarily analyzed against each tested mould. Plates of malt extract agar were supplemented with increasing concentrations of untreated CFS from *L. plantarum* cultures at stationary phase, according to Wang et al. (2012). No inhibition was detected at the highest concentration of CFS (12% v/v) of the strains that in the previous screening had not shown antagonistic activity against a target filamentous fungi (data not shown). Similarly, when *L. plantarum* were classified as mild inhibitor strains, a concentration of 8% (v/v) was required to observe a reduction in the growth of the mould (data not shown). Finally, CFS from *L. plantarum* with strong inhibitory activity

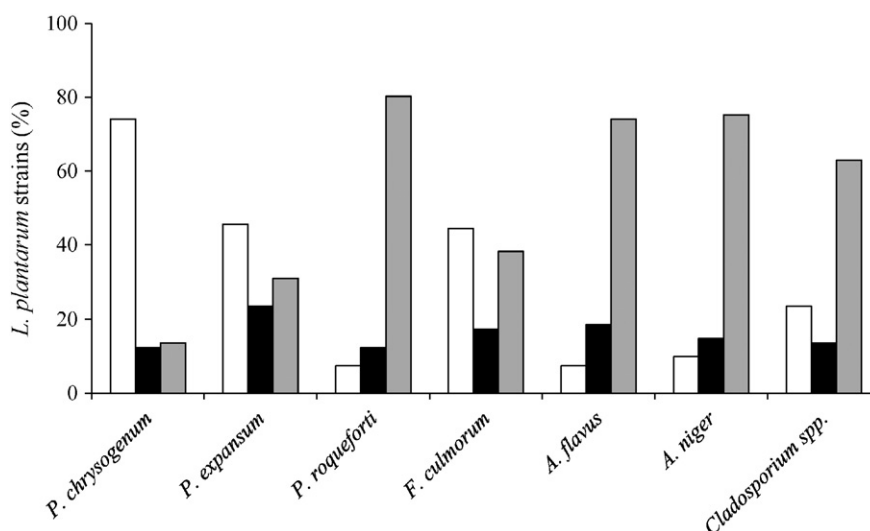


Fig. 1. Antifungal activity of *L. plantarum* strains by the overlaid method. *L. plantarum* strains showing (%) strong (white bar), mild (black bar) or no (gray bar) inhibition against the tested fungal species using the overlaid method.

Table 1

Antifungal activity of *L. plantarum* strains with broad spectrum. *L. plantarum* strains showing broad antifungal spectrum activity against the tested fungal species. Strains were classified as no (\pm), mild (+), or strong (++) inhibitors by using the overlayed method. Experiments were repeated three times.

<i>L. plantarum</i>	<i>P. chrysogenum</i>	<i>P. expansum</i>	<i>P. roqueforti</i>	<i>F. culmorum</i>	<i>A. flavus</i>	<i>A. niger</i>	<i>Cladosporium</i> spp.
UFG 10	+	++	+	++	\pm	\pm	++
UFG 94	++	\pm	+	+	\pm	\pm	++
UFG 96	++	++	\pm	\pm	+	++	\pm
UFG 97	++	\pm	+	++	\pm	\pm	++
UFG 98	++	\pm	++	+	\pm	\pm	++
UFG 108	++	++	\pm	++	+	\pm	+
UFG 115	++	\pm	+	++	\pm	\pm	++
UFG 120	++	++	+	\pm	\pm	++	\pm
UFG 121	+	++	++	++	+	\pm	\pm

Table 2

Hyphal radial growth inhibition by CFS from *L. plantarum* UFG 108 and *L. plantarum* UFG 121. Hyphal radial growth inhibition after 3 and 5 days of incubation at 24 °C in plates of malt extract agar not (control) or supplemented with CFS (12% v/v) from culture at stationary phase of *L. plantarum* UFG 108 and *L. plantarum* UFG 121. Results are expressed as the hyphal diameter (mm) and the corresponding percentage of inhibition.

	<i>P. chrysogenum</i>	<i>P. expansum</i>	<i>P. roqueforti</i>	<i>F. culmorum</i>	<i>A. flavus</i>	<i>A. niger</i>	<i>Cladosporium</i> spp.
3 days							
Control	1.6	2.8	2.2	2.8	2.2	2	1.7
UFG 108	1.2 (25%)	1.7 (39%)	2.2 (0%)	1.2 (57%)	1.7 (23%)	1.9 (5%)	1.4 (18%)
UFG 121	1.4 (13%)	1.8 (36%)	2 (9%)	1.2 (57%)	1.5 (32%)	1.7 (15%)	1.2 (29%)
5 days							
Control	3.4	8.5	6.7	8.5	5	4	1.8
UFG 108	2.3 (32%)	4.1 (52%)	6.6 (1%)	3.2 (62%)	3.8 (24%)	3.7 (8%)	1.5 (17%)
UFG 121	2.7 (21%)	4 (53%)	4.2 (37%)	3.4 (60%)	3.6 (28%)	3.5 (13%)	1.5 (17%)

was able to inhibit the growth of a fungal strain when supplemented at 4% (v/v) in the media, and the hyphal radial growth inhibition was higher increasing the CFS concentrations (data not shown). However, none of the *L. plantarum* strain tested was able to completely inhibit the growth of the analyzed filamentous fungi. In particular, 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* CECT 2278 and *F. culmorum* CECT 2148 was reduced of about 50% and 60%, respectively (Table 2). Results of the inhibition detected after 3 and 5 days of incubation with CFS (12% v/v) from both strains are also reported for all the tested moulds (Table 2).

3.3. Analysis of active compounds in CFS

With the aim to characterize the active antifungal compounds, the cell-free supernatant from cultures of *L. plantarum* UFG 108 and *L. plantarum* UFG 121 was further investigated by well diffusion agar, according to Al Kassaa et al. (2014). Thus, CFSs were submitted to serial physical and chemical treatments that allowed to exclude or to confirm the chemical nature of the antifungal molecules. All treated CFSs were tested against each target filamentous fungi. The results revealed that CFSs lost their antagonistic properties only after pH neutralization (data not shown), suggesting that the inhibition ability was due to the occurrence of organic acids associated with the low pH.

Therefore, the active fraction CFS-A was analyzed by HPLC at different times of growth of the *L. plantarum* UFG 108 and UFG 121. An exponentially increasing concentration of lactic acid produced by both *L. plantarum* cultures was found throughout the incubation time. Thus, 5 g L⁻¹ of lactic acid were detected after 6 h of incubation that doubled in the next 2 h, until a maximum value of about 25 g L⁻¹ after 24 h (Fig. 2), suggesting that the CFS from stationary phase cultures could be more effective against moulds than CFS from exponentially growing cultures. Similarly, the highest amount of phenyllactic acid (around 0.06 g L⁻¹) was found after 24 h (data not shown). According to this, when CFSs obtained from *L. plantarum* UFG 108 and UFG 121, 8 h cultures were incorporated in plates of malt extract agar at a concentration of 12%, no inhibitory effect was observed against the target mould

F. culmorum after 5 days (Fig. 3). In contrast, at the same conditions, the CFSs recovered after 24 h cultures were able to reduce the growth of *F. culmorum* by more than 60% (Fig. 3).

3.4. Inhibition of *F. culmorum* on fermented oat-based products

A new fermented oat-based product was obtained according to Russo et al. (2016) by inoculating as a starter culture the strongest antifungal strain, i.e. *L. plantarum* UFG 121, at a concentration of 8×10 (Coda et al., 2012) cfu g⁻¹. After a fermentation step of 16 h, the microbial load of the starter increased up to 2×10 (Cortés-Zavaleta et al., 2014) cfu g⁻¹ (data not shown), confirming that the oat matrix was a suitable substrate for the microbial growth. At this time, oat products were submitted or not to a thermal treatment in order to investigate the antifungal effectiveness of live microorganisms rather than of the metabolites produced during the fermentation process. The main qualitative attributes and microbial viability of both control samples and artificially contaminated samples with *F. culmorum* were monitored over 21 days, a time corresponding to a reasonable shelf life of this kind of

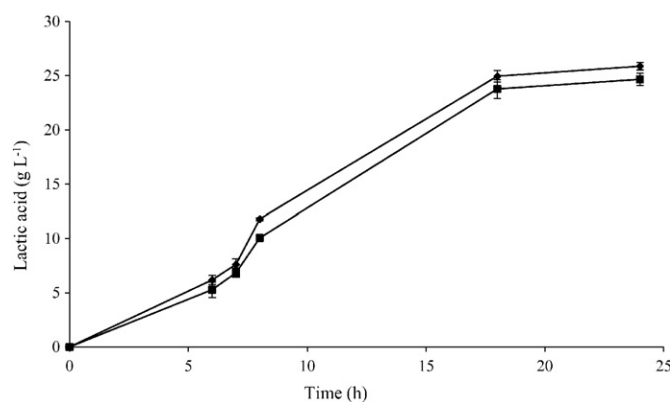


Fig. 2. Lactic acid production of *L. plantarum* strains with broad antifungal spectrum activity. Lactic acid (g L⁻¹) production by *L. plantarum* UFG 121 (diamond) and *L. plantarum* UFG 108 (square) after 6, 7, 8, 18, and 24 h of incubation at 30 °C.

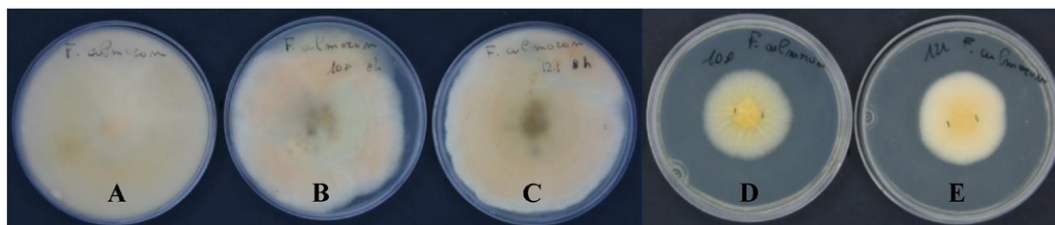


Fig. 3. Hyphal radial growth inhibition of *F. culmorum* CECT 2148 by CFS from *L. plantarum* UFG 108 and *L. plantarum* UFG 121 at different growth phase. Inhibition of *F. culmorum* CECT 2148 after 5 days of incubation at 24 °C on plates of malt extract agar supplemented with 0% (A), and 12% of CFS from *L. plantarum* UFG 108 (B) and *L. plantarum* UFG 121 (C) after 8 h, or from *L. plantarum* UFG 108 (D) and *L. plantarum* UFG 121 (E) after 24 h of incubation at 30 °C.

products. In unpasteurized samples, the microbial viability of *L. plantarum* UFG 121 decreased after the first week of storage to about 8×10 (Coda et al., 2012) cfu g^{-1} , without any substantial changes until the end of the shelf life (Fig. 4). No differences were observed in samples contaminated with *F. culmorum* (Fig. 4). As expected, a concentration lower than 10 (Axel et al., 2015) cfu g^{-1} of *L. plantarum* UFG 121 was detected in samples submitted to the thermal stabilization (data not shown).

As reported in Fig. 5, we were unable to observe signals of deterioration of the product at the end of the shelf life in unpasteurized samples (Fig. 5A and Fig. 6). Interestingly, a lack of significant alterations was also observed in unpasteurized samples which had been artificially inoculated with spores of *F. culmorum* (Fig. 5B and Fig. 6). Similarly, no alteration was detected in samples fermented by *L. plantarum* UFG 121 and submitted to thermal stabilization (Fig. 5C and Fig. 6). By contrast, pasteurized samples inoculated with *F. culmorum* showed a drop in the level of some of the analyzed quality attributes after three weeks of cold storage, especially in terms of reduction of viscosity (Fig. 5D and Fig. 6). Control, unfermented samples, artificially inoculated with *F. culmorum* were considered below the limit of marketability after only 7 days of storage at 4 °C, mainly due to the development of the mould that seemed to negatively affect the rheological features of the product. The overall appearance of these samples was considerably deteriorated along storage, resulting in a clotted, inhomogeneous, mouldy product (Fig. 5E). Moreover, a high production of off flavors and off-odors was detected in these samples (Fig. 6).

4. Discussion

Filamentous fungi are widespread food spoilage microorganisms responsible for significant economic loss in the food industry, as well as related to critical safety concerns due to their potential ability to produce mycotoxins. In the last years, lactic acid bacteria have been extensively investigated for their antifungal properties and bioprotective

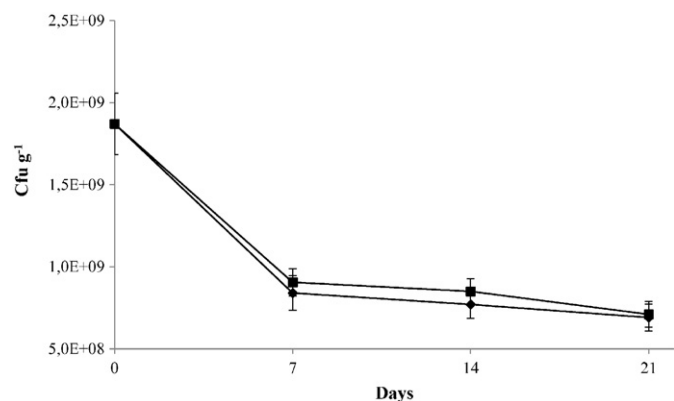


Fig. 4. Survival of *L. plantarum* UFG121 in fermented oat-based products during storage. Microbial population of *L. plantarum* UFG 121 (cfu g^{-1}) in fermented oat-based products containing live *L. plantarum* UFG 121 (square) and artificially contaminated with *F. culmorum* CECT 2148 (diamond) after 7, 14, and 21 days of storage at 4 °C.

cultures have been proposed as promising biotechnological approaches for the food sector (Pawlowska et al., 2012; Ryan et al., 2011). It is known that *L. plantarum* have become prominent players in the field of antifungal research among members of the *Lactobacillus* genus (Crowley et al., 2013b). However, at the best of our knowledge, this is the first work focused on the screening of a collection of only *L. plantarum* strains isolated from different food matrices for their antifungal activity. Moreover, according to the development of innovative novel foods in the

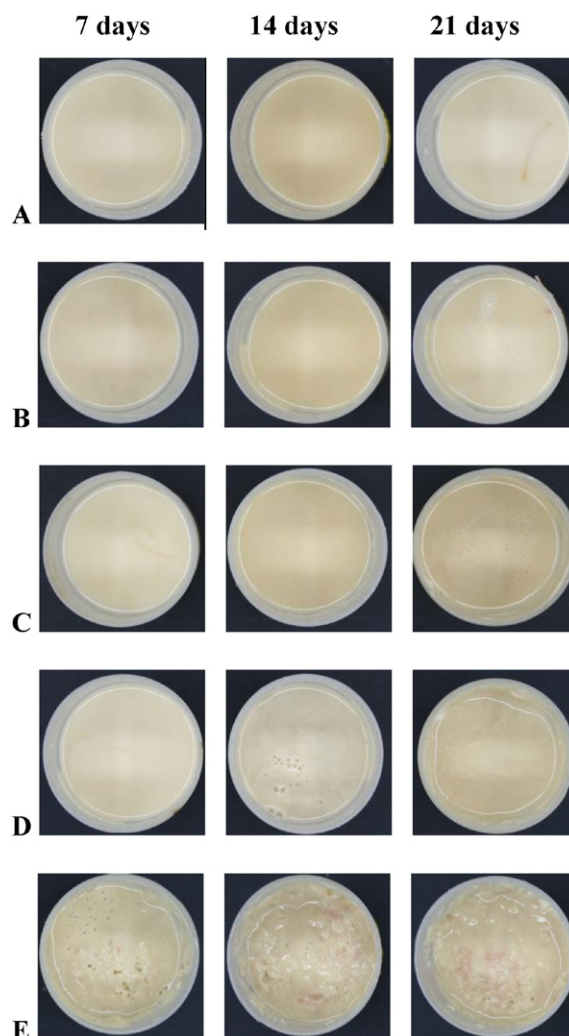


Fig. 5. Image acquisition of fermented oat-based products during the storage. Fermented oat-based products containing live *L. plantarum* UFG 121 (A, B) or submitted to thermal treatment after the fermentation step (C, D), not (A, C) or artificially contaminated with *F. culmorum* CECT 2148 (B, D) after 7, 14, and 21 days of storage at 4 °C. Control (E) was not fermented by *L. plantarum* UFG 121 and artificially inoculated with *F. culmorum* CECT 2148.

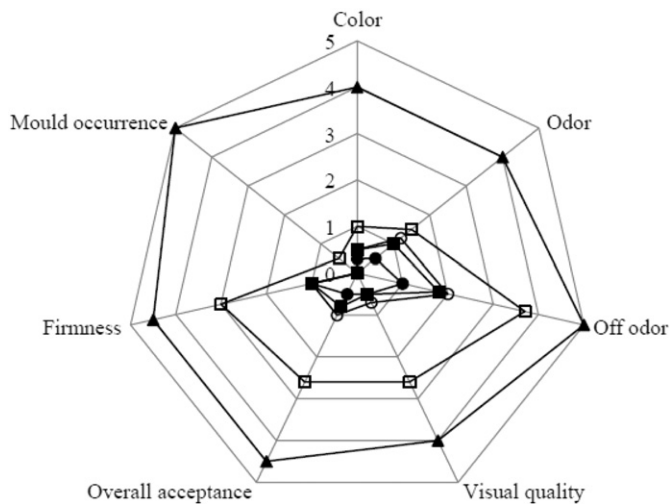


Fig. 6. Sensorial evaluation of fermented oat-based products. Sensorial evaluation of oat-based products containing live *L. plantarum* UFG 121 (black circle) and artificially contaminated with *F. culmorum* CECT 2148 (white circle), thermal treated (black square) and artificially contaminated with *F. culmorum* CECT 2148 (white square), or not fermented with *L. plantarum* UFG 121 and artificially contaminated with *F. culmorum* CECT 2148 (dashed line) after 21 days of storage at 4 °C. Every descriptor was scored on a 1 to 5 scale, where 1 = typical/highest perception of quality; 5 = atypical/lowest perception of quality.

market, the antifungal ability of the best antagonist was investigated by using as model a new fermented oat-based product.

Here, the antifungal potential of eighty-eight *L. plantarum* isolates was preliminary screened against seven moulds that are typical inhabitants of cereal based matrices. Although high levels of fungal contamination can be found from crop farming to cereal products, after processing the main spoilage fungi belong to the genera *Aspergillus*, *Penicillium*, *Fusarium*, and *Cladosporium* (Oliveira et al., 2014). In this work, a great interspecific variability was observed considering the antifungal activity spectrum of each *L. plantarum*. However, *P. chrysogenum*, *P. expansum*, and *F. culmorum* were found as the more sensitive species. In contrast, *P. roqueforti* was the most resistant one. According to these findings, in a recent screening none of about 900 LAB tested showed strong inhibition ability against *P. roqueforti* (Cheong et al., 2014). In the same study, twelve LAB isolates that displayed antifungal activity against species belonging to the genera *Aspergillus*, *Penicillium* and *Cladosporium* were all identified as belong to *L. plantarum* species (Cheong et al., 2014). Similar results were reported by Magnusson et al. (2003) that analyzing 1200 LAB isolates unveiled several strains with strong inhibitory activity against *Aspergillus*, *Penicillium*, and *Fusarium* spp., but not against *P. roqueforti*. Recently, a large scale screening on about 7000 presumptive LAB revealed that the majority of *L. plantarum* was able to inhibit the growth of *P. expansum* (Crowley et al., 2012a). In general, *L. plantarum* was shown to be active against spoilage filamentous fungi typically found in baked-goods such as *Penicillium* spp. and *Fusarium* spp. (Dal Bello et al., 2007; Gerez et al., 2010).

LAB antimicrobial activity is expressed either directly through the competition of the live cells for growth substrates, or indirectly as the result of the synthesis of a wide variety of active antagonistic metabolites. Therefore, CFSs obtained from culture at stationary phase of *L. plantarum* isolates showing the broad antifungal activity spectrum were submitted to further investigation. The incorporation of different concentrations of CFS into agar plates for fungal growth is a fast method to establish the anti-mycotic potential of LAB cultures. Recently, Wang et al. (2012) reported that CFS from *L. plantarum* IMAU10014 was able to completely inhibit the growth of the plant pathogen *Phytophthora drechsleri* when added at a concentration of 8–10% (v/v) to the media, while the corresponding minimal inhibitory concentration was 3% (v/v). On the basis of similar results, in the present study the CFSs of the

L. plantarum strains showing higher inhibition (50–60% against *P. expansum* and *F. culmorum*) were analyzed in order to characterize the major antifungal compounds. Antifungal compounds of *L. plantarum* have previously been purified and characterized (Lavermicocca et al., 2000; Prema et al., 2010; Ryu et al., 2014; Sjögren et al., 2003; Wang et al., 2012; Yang et al., 2011).

The results obtained suggested that the antifungal activity was due to the production of organic acids. This hypothesis was confirmed by HPLC analysis that showed the occurrence of high concentration of lactic acid in the CFSs. Among organic acid, lactic, acetic and phenyllactic acids were identified as the main responsible for antifungal effect (Lavermicocca et al., 2003; Gerez et al., 2010). In contrast, lactic acid was found exhibit a very weak inhibitor potential in comparison to other organic acid such as phenyllactic acid or formic acid (Rizzello et al., 2011).

Interestingly, it was found a higher production of lactic acid in the CFS from culture after 24 h of growth, that matched to the maximal antifungal activity displayed. Accordingly, Sathe et al. (2007) reported that by using MRS broth as culture media, the antifungal activity of CFS from *L. plantarum* was growth-dependent and was highest at the end of logarithmic phase of growth while declines during the late stationary phase. However, a maximal production of antifungal compounds was reported during stationary phase suggesting the occurrence of secondary metabolites that could exert complex interaction with synergistic effect (Magnusson and Schnurer, 2001).

In the food industry the employment of antifungal LAB ‘factories’ as biopreservatives agents is a concrete and exciting challenge. In the last years, efforts have been mainly addressed with successful outcomes to improve the shelf life of bakery products (Coda et al., 2011; Gerez et al., 2010, 2013). Recently, some attempts to introduce LAB with antifungal activity into the food chain have been also proposed in the field of dairy products (Crowley et al., 2012b; Cheong et al., 2014), or for fruit and vegetables (Crowley et al., 2012a). However, this biotechnological approach is still an unexplored field for the elaboration of innovative novel foods.

In a recent work, we produced and characterized from a technological point of view a new functional oat-based product with the aim to simultaneously provide the beneficial effects of probiotic microorganisms, oat and its components such as their soluble fibers, i.e. β -glucan (Russo et al., 2016). In the present study, *L. plantarum* UFG 121 was investigated for its biopreservative potential on samples artificially contaminated with *F. culmorum*. This filamentous fungi was selected as indicator mould due to its common occurrence as spoilage on cereals-based foods (Mauch et al., 2010; Oliveira et al., 2012; Ryan et al., 2011). The ability to fight the fungal spoilage was evaluated in terms of inhibition of any visible growth of fungal mycelium on the product surface. In particular, two approaches were explored based on the application or not of a thermal treatment after the fermentation step. The first approach was compatible with the production of a microbiologically stabilized food naturally enriched in antimicrobial compounds during the fermentation process. In this regard, fermentation of sourdoughs with starter cultures showing antifungal properties was proposed as a strategy to remarkably enhance the shelf life of bakery products (Coda et al., 2011; Garofalo et al., 2012). Accordingly, it was reported that antifungal compound(s) from *L. plantarum* are heat stable at temperatures of 121 °C for 15 min leading to potential application of the metabolites prior to pasteurization (Crowley et al., 2012b). In contrast to heat-stabilized samples, the second approach was characterized by high concentrations of *L. plantarum* UFG 121. This treatment provided the best biopreservative effect, since no differences were observed in terms of some qualitative features between not or contaminated with *F. culmorum* samples until the end of the shelf life. Nonetheless, samples submitted to a thermal treatment were able to increase the shelf life of the oat-based product at least until the second week of storage, indicating that a strong bioprotection could be provide even by

antifungal compounds produced during a long fermentation step. These results suggested that the production of antifungal metabolites could increase during the storage time or that different competition phenomena could occur if live bacteria were present in the food.

In conclusion, we confirmed that the employment of LAB with antifungal activity is a valuable approach to fight against the development of spoilage filamentous fungi in an oat-based food model. In particular, if live antagonistic LAB are vehicle with the food they could provide a strong biopreservation with positive effect on the shelf life and on the safety of the product. These findings, could provide new insights for biotechnological application in the field of functional foods supplemented with live biopreservative microbes post heat treatment or for the elaboration of new probiotic foods, as well as they would be useful to test LAB strains cocktails to inhibit resistant moulds.

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