Functional characterization of *Lactobacillus plantarum* ITEM 17215: a potential biocontrol agent of fungi with plant growth promoting traits, able to enhance the nutritional value of cereal products

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ABSTRACT

In this work, we explored the potential of 25 *Lactobacillus plantarum* strains isolated from cereals and milk-based products, testing characteristics related to antifungal activity and to nutritional quality. The tested strains demonstrated interesting beneficial traits, such as the ability to utilize fructo-oligosaccharides, prebiotic substances that help probiotic microorganisms to grow in the human gut, and to reduce phytate, an antinutrient present in cereal sector. Regarding mould inhibition, we highlighted the ability of the strains to inhibit *Penicillium roqueforti, Mucor circinelloides* and mycotoxinogenic moulds associated with cereal grains as Aspergillus *flavus, A. niger, Fusarium verticillioides.* Moreover, a moderate reduction of the bioavailability of aflatoxin AFB1 was detected. The selected *L. plantarum* strain ITEM 17215, showed a strong inhibitory ability towards fungal growth and was able to produce 1,2-dihydroxybenzene, benzoic acid, p-hydroxyphenyllactic acid and 3-phenyllactic acid. The latter compound, already described as efficient antifungal inhibitor, was the most abundant and its concentration was further increased by adding phenylalanine and phenylpyruvic acid in the growth medium. The metabolites produced by strain ITEM 17215 could also be related to the ability of the strain to induce cereal germination and promote plant growth. This aspect, not yet investigated in *L. plantarum*, could have interesting applications in the agro-food sector.

Keywords: Lactobacillus plantarum, antifungal activity, 3-phenyllactic acid, aflatoxin, plant-growth promoting activity, phytate reduction, cereal sector

Chemical compounds studied in this article

3-phenyllactic acid (PubChem CID: 3848), p-hydroxyphenyllactic acid (PubChem CID: 9378), 1,2-dihydroxybenzene (PubChem CID: 289), benzoic acid (PubChem CID: 243), lactic acid (PubChem CID: 612), acetic acid (PubChem CID: 176), phenylalanine (PubChem CID: 6140), phenylpyruvic acid (PubChem CID: 997), aflatoxin B1 (PubChem CID: 186907)

1. Introduction

With the aim of improving the healthiness and quality of food, in the last decade, a great attention has been paid to the possibility of adopting bio-control strategies, through a targeted use of food grade microorganisms capable of enhancing the nutritional quality and controlling the development of pathogenic microorganisms along the whole chain, without interfering with the process itself (Dalié, Deschamps, & Richard-Forget, 2010). This approach should reduce not only the growth of undesirable microorganisms and the amount of their toxic metabolites, but should also reduce substantially the use of chemicals and preservatives (towards which, among other substances, several microorganisms have acquired resistance properties).

In fermented foods, Lactic Acid Bacteria (LAB) are the main protagonists, both in terms of use and commercial value. Recently, through extensive programs of screening, new LAB biotypes, isolated from different ecological niches and food matrices, have been studied. Some of these strains, showing high performance and competitiveness, are used as probiotics (Hill et al., 2014) for the development of new food formulations with functional characteristics (Batista et al., 2017; Lollo et al., 2015) and for the control of the growth of undesirable microorganisms. It refers, for example, to the antifungal properties of strains of *Lactococcus* spp. and *Lactobacillus* spp. (Crowley, Mahony, & van Sinderen, 2013; Stoyanova, Ustyugova, & Netrusov, 2012), and to another promising bio-control strategy, linked to the ability of selected strains to interact with preformed mycotoxins, reducing the bioavailability of the toxic compounds (Bovo Campagnollo et al., 2016; El-Nezami, Kankaanpa, Salminen, & Ahokas, 1998; Meca, Ritieni, & Mañes, 2012; Peltonen, El-Nezani, Haskard, Ahokas, & Salminen, 2001; Zhu, Hassan, Watts, & Zhou, 2016). Investigations about the ability of LAB to remove mycotoxins suggested that binding to cell wall components is more likely to be the mechanism of removal, rather than metabolic processes. (Dalié et al., 2010; Shetty & Jerspersen, 2006). Recent studies, in dairy sector mainly, have shown that selected probiotic strains are able to remove aflatoxin from contaminated milk and during yoghurt production and storage (Elsanhoty, Salam, Ramadan, & Badr, 2014; Sarlak et al., 2017; Serrano-

Niño et al., 2013). Studies regarding the use of probiotic strains as detoxification agents in other food sectors are still limited.

A major issue in the cereal sector was and still is the deterioration of the raw material by filamentous fungi. The fungal growth causes not only significant economic losses but also has important implications for food safety. Indeed, fungal contamination can cause loss of a whole grain stock, as well as an accumulation of mycotoxins, toxic secondary metabolites of which synthesis is favoured at high temperatures and humid conditions (Ahlberg, Joutsjoki, & Korhonen, 2015; Błajet-Kosicka, Twarużek, Kosicki, Sibiorowska, & Grajewski, 2014; Neme & Mohammed, 2017). Among mycotoxins, aflatoxins (AF) and fumonisins (F) are one of the most dangerous mycotoxin found in cereals. The aflatoxin AFB1, produced by *Aspergillus flavus* and *A. parasiticus*, is the most carcinogenic of the natural occurring aflatoxins (Strosnider et al., 2006). The genus *Fusarium* is known for the ability of synthesizing several mycotoxins, among which deoxynivalenol, produced by *F. graminearum* (Fung & Clark, 2004), and fumonisin B1 produced by *F. verticillioides* frequently found in cereals, particularly in maize (Deepa & Sreenivasa, 2017). This constitutes a relevant health problem in countries where maize is the staple food (van Rensburg, McLaren, Flett, & Schoeman, 2015). A lack of attention in the application of good agricultural practices is the main reason for accumulation of mycotoxins in raw materials during harvest and storage (Neme & Mohammed, 2017). These toxins, through the production chain, can easily come to the final consumer, with great health risks.

Another aspect related to cereal consumption is the presence of anti-nutritional factors. Among these factors, phytate is the most important, because it has strong ability to complex multi-charged metal ions (Coulibaly, Kouakou, & Chen, 2011).

Multiple publications have highlighted the positive properties of LAB strains in cereal sector. Within LAB group, *Lactobacillus plantarum* is considered one of the most suitable protective microorganisms in cereal production chain. *L. plantarum* is a versatile and competitive species, adapting to different environmental conditions. These features can explain its presence, as a

105 member of the complex microbiota, in many sourdoughs (De Vuyst et al., 2014). The growth
106 inhibition of fungi is mainly related to the production of organic compounds during fermentation
107 metabolism. The interest dedicated to *L. plantarum* is related to the ability of producing
108 antimicrobial compounds, especially antifungal metabolites, among which 3-phenyllactic acid
109 (PLA), 4-hydroxyphenyllactic acid (Cortés-Zavaleta, López-Malo, Hernández-Mendoza, & García,
110 2014; Lavermicocca, Valerio, & Visconti, 2003; Poornachandra Rao et al., 2017; Russo et al., 2016;
111 Zhang, Zhang, Shi, Shen, & Wang, 2014) and cyclic dipeptides (Dal Bello et al., 2007), the
112 production of which in cereal-based products increases their safety and shelf-life (Dalié et al., 2010;
113 Oliveira, Zannini, & Arendt, 2014; Russo, Fares, Longo, Spano, & Capozzi, 2017). Among them,
114 PLA, derived from the phenylalanine catabolism, has raised a noticeable interest (Chaudhari &
115 Gokhale, 2016; Valerio, Lavermicocca, Pascale, & Visconti, 2004). This compound has been also
116 related to growth promotion of rice (*Oriza sativa*), increasing water and nutrient absorption and
117 seed endosperm utilization efficiency (Adachi et al., 2013). The antimicrobial mechanism of action
118 of PLA is still unclear. However, the possibility to enhance its production in LAB by adding
119 suitable precursors has been reported (Li, Jiang, & Pan, 2007; Valerio, Di Biase, Lattanzio, &
120 Lavermicocca, 2016).
121 This study aimed to be a further exploration of the potential of the species *L. plantarum*, through
122 the evaluation of properties not yet deepened: (i) the ability of inhibition *in vitro* and *in situ* of
123 moulds not investigated up to now, (ii) the aflatoxin binding, (iii) the potential to induce cereal
124 germination and to promote plant growth, (v) the phytate degradation ability and fructo-
125 oligosaccharides utilization. Moreover, studies have been carried out on antimicrobial compounds
126 produced by the selected *L. plantarum* ITEM 17215, and on the conditions influencing the PLA
127 production.
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130 **2. Materials and methods**

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132 *2.1. Bacterial strains and culture conditions*

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134 Twenty-five *Lactobacillus plantarum* strains were studied. The strains were previously isolated
135 from different sources:CR62, CR63 CR67, CR70, CR71, CR73, CR74, CR75, CR77, CR78 from
136 maize bran, CE42, CE60, CE84 (deposited in the Agro-Food Microbial culture Collection of the
137 Institute of Sciences of Food Production, CNR, Bari, Italy as ITEM 17215) from wheat bran, 93,
138 MD143, MD123, MD147, VS516, VC114, VC194, VC233, SE90, SE140 from raw milk cheeses,
139 AC, LC1 from cocoa beans. *Lactobacillus rhamnosus* strain GG (ATCC 53103) was used as a
140 positive control in aflatoxin binding experiments. Strains were routinely grown in anaerobic
141 conditions (Anaerocult A, Sigma, St Louis, MO, USA) in MRS broth/agar (Difco Lab., Augsburg,
142 Germany), for 24-48 h at 30 °C.
143 Growth in milk was studied using 9% RSM (Reconstituted Skim Milk- Difco) incubated at 30
144 °C. For studies of 3-phenyllactic acid (PLA) production, MRS broth and RSM, supplemented with
145 1.5 g L^{-1} phenylalanine (Phe) (Sigma) and 3.0 g L^{-1} phenylpyruvic acid (PPA) (Sigma) were used.
146 Bacterial concentration was measured as CFU mL^{-1}. Acidifying activity of the strains was tested in
147 MRS broth; the pH was measured and recorded automatically, throughout the 48 h incubation
148 period.

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150 *2.2. Fungal strains and culture conditions*

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152 *Fusarium verticillioides* FSq1, *Aspergillus niger* FS11, *Aspergillus flavus* FS13, *Mucor*
153 *circinelloides* FSq2 and *Penicillium roqueforti* FS 22 (from the Collection of the Department of
154 Health, Animal Science and Food Safety, University of Milan, Italy) were routinely grown on Malt
155 Extract Agar (MEA) (Merck, Darmstadt, Germany) at 25 °C for 5-7 days and stored at 4°C until
156 further use. Fungal spore suspensions were harvested by adding 15 mL of sterile milli-Q water, and

stored at 4° C until further use. Concentration of the spores was assessed by flow cytometer estimation (BD Accuri C6 Flow Cytometer, BD Biosciences, Franklin Lakes, NJ USA).

2.3. Antifungal activity in vitro

Antifungal activity of the *L. plantarum* strains was investigated with an overlay assay (Axel et al., 2016). Bacteria, grown for 16 h in MRS broth at 30 °C, were inoculated in 2-cm lines on MRS agar plates and allowed to grow anaerobically for 48 h at 30°C. Subsequently, plates were overlaid with 10 mL of cooled soft (0.7%) MEA containing mould spore suspension (10^4 spores mL^{-1}). Plates were incubated for 4 days at 25°C, and the antifungal activity was evaluated as clear zones of inhibition around the bacterial smears. The degree of inhibition was calculated on the basis of the inhibition zone as no inhibition (-) for inhibition zone smaller than 3 mm, moderate (+/-) for inhibition zone ranging from 3 to 10 mm or strong (+) for inhibition zone larger than 10 mm. The typical aspect of the inhibition halos is shown in Fig. 1.

Antifungal activity was also tested on cell-free supernatant (CFS): *L. plantarum* strains were inoculated in MRS broth to an initial concentration of 10^6 CFU mL^{-1} and incubated at 30°C for 48 h. CFS was obtained by centrifugation (3000 x *g*, 5 min) and sterilized by filtration (0.2 μm pore-size cellulose acetate filter). Plates of MEA containing 10^4 mould spores mL^{-1} were supplemented with different concentrations of CFS (10%-50%-80% v/v), incubated at 25 °C for 5 days and subsequently examined for inhibitory activity against *F. verticillioides, M. circinelloides* and *A. flavus.*

2.4. Antifungal activity in cereals and milk-based products

L. plantarum strains that displayed the highest antifungal activity were further tested for their ability to inhibit the moulds during cereal storage in simulated micro-silos and micro-yogurt preparations.

A micro-silo consisted of a test tube (3 x 11 cm) containing 20 g of a compacted mix of cereals (wheat, rice and oat in equal proportion) pre-treated with 20% of water for 48 h at 4°C. In one micro-silo the mixture was co-inoculated with 10^4 spores g^{-1} of *F. verticillioides* or *M. circinelloides*, or *A. flavus* and 10^6 CFU g^{-1} of *L. plantarum.* Another micro-silo, inoculated only with mould spores, was used as a control. The micro-silos were placed at room temperature (comprised between 19 and 22 °C) and mould growth was daily monitored for 10 days.

For micro-yogurt preparation, 20 mL of 9% RSM were inoculated with commercial starter cultures of *Streptococcus thermophilus* and *L. delbrueckii* subsp. *bulgaricus* (15 mg L^{-1}) (Lyofast – Sacco System, Cadorago, Italy) and 10^6 CFU mL^{-1} of *L. plantarum*. Fermentation was performed at 43 °C for 5-6 h, until the pH reached a value of 4.7. Subsequently, the resulting micro-yogurt preparations were transferred into petri plates, inoculated with *P. roqueforti* to a final concentration of 10^4 spores mL^{-1} and incubated at 25 °C. Samples were analysed for mould growth inhibition after 7 days of incubation. Yogurt without adding *L. plantarum* was used as negative control.

2.5. Effect of L. plantarum *on growth rate of* A. flavus*,* M. circinelloides *and* F. verticillioides

Twenty five mL of malt extract broth in 100 mL Erlenmeyer flask were inoculated with 10^9 CFU mL^{-1} of overnight *L. plantarum* cultures and 10^4 mould spores mL^{-1}. Fungal mycelial mass, harvested after different incubation times at 25°C, was separated by filter paper and weighed after drying at 105°C.

2.6. Identification of targeted antifungal compounds

L. plantarum strain ITEM 17215 was grown in MRS broth at 37 °C for 48 h. CFS was ultrafiltered (1kDa) and subsequently purified using the Quick, Easy, Cheap, Effective, Rugged and Safe (QuEChERS) procedure, as described by Brosnan, Coffey, Arendt, & Furey (2014a) using a dispersive solid phase extraction (dSPE) kit (Agilent Technologies, Santa Clara, CA, USA). According to the procedure reported by Brosnan, Coffey, Arendt, & Furey (2012) and Brosnan, Coffey, Arendt, & Furey (2014b), purified extracts were subjected to the Ultra Performance Liquid Chromatography - Photo Diode Array - High Resolution - Tandem Mass Spectrometry (UPLC/PDA-ESI-HR-MS/MS) analysis for targeted identification of 16 potential antifungal compounds including 1,2-dihydroxybenzene, allyl phenylacetate and the following acids: DL-p-hydroxyphenyllactic, 4-hydroxybenzoic, 3,4-diydroxyhydrocinnamic, vanillic, caffeic, 3-(4-hydroxyphenyl) propionic, PLA, (E)-p-coumaric, 3-(4-hydroxy-3-methoxyphenyl), propanoic, benzoic, (E)-ferulic, salicylic, hydrocinnamic and α-methylcinnamic. The UPLC-PDA-HR-MS/MS analyses were carried by coupling an Acquity UPLC separation module (Waters, Milford, MA, USA), equipped with a Gemini C18 column (150 x 2.0 mm. 3 µm, 110 Å, Phenomenex, Torrance, CA, USA) to an Acquity PDA eλ Detector (Waters) and (in-line) a Q Exactive hybrid quadrupole-Orbitrap mass spectrometer through a HESI-II probe for electrospray ionisation (Thermo Fisher Scientific, San Jose, CA, USA). Data were processed using the Xcalibur software (version 3.0, Thermo Fisher Scientific).

2.7. Quantification of organic acids

3-Phenyllactic, lactic and acetic acids were quantified through an HPLC system (L 7000, Merck Hitachi) equipped with RI and UV (210 nm) detectors serially connected, using a SH1821 column (300 x 8 mm, Shodex, München, Germany) maintained at 50 °C and eluted with 5 mM H_2SO_4 at 0.5 mL min^{-1}. Analytical grade organic acids were used as standards (Sigma). Four-point external

232 calibration curves ($R^2 > 0.998$) were adopted to quantify PLA, acetate, and lactate in MRS broths.
233 LOQ of 0.010 g L^{-1} and LOD of 0.005 g L^{-1} (S/N = 3) for PLA were obtained, respectively.
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235 *2.8. Determination of MIC values*
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237 The MIC values of PLA, lactic acid and acetic acid were determined. Dilution of each acid, with
238 concentration range between 0.5 and 7.5, 6.0 and 50.0, 0.1 and 3.0 mg mL^{-1} for PLA (Sigma), lactic
239 acid (Sigma) and acetic acid (Sigma) respectively, were added to MEA medium inoculated with 10
240 spores mL^{-1} of *A. flavus*, *M. circinelloides* or *F. verticillioides.* MIC was defined as the lowest
241 concentration where no growth could be observed after the incubation period.
242
243 *2.9. Aflatoxin binding assay*
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245 Aflatoxin binding assay was carried out as described by Haskard, El-Nezami, Kankaanpa,
246 Salminen, & Ahokas (2001). Solid AFB1 (Sigma) was suspended in benzene-acetonitrile (97/3;
247 v/v) to obtain an AFB1 concentration of approximately 2 mg mL^{-1}. A solution of 5 µg mL^{-1} AFB1
248 was prepared in PBS (pH 7.3), and the benzene-acetonitrile was evaporated by heating in a water
249 bath (80 °C, 5 to 10 min). For each bacterial strain, a volume of the culture broth corresponding to
250 10^{10} cells was centrifuged (3000 x *g*, 15 min, 10 °C) and the bacterial pellets were washed with 5
251 mL of Milli-Q water, suspended in 1.5 mL of AFB1 solution (5µg mL^{-1}) and incubated at 37°C.
252 After 24 h of incubation, the bacteria were pelleted (3000 x g, 10 to 15 min, 10°C), and the
253 supernatant (200 µL) containing AFB1 was collected and stored at -20 °C. For each strain, a
254 bacterial control (bacteria suspended in PBS) and an AFB1 control (5 µg mL^{-1} of AFB1 in PBS)
255 were tested. For strains showing the highest binding ability, the effect of incubation time was
256 evaluated at 0, 24 and 48-h time points. The analysis was carried out on HPLC system consisting of
257 two 1580 HPLC pump (Jasco, Easton, MD, USA), a degaser unit DG 2080-53 (Jasco), a FP 1520

fluorimeter detector set up at 365 nm excitation and 415 nm emission, a manual sampler with a loop of 20 µL (Rheodyne). The analyses were performed isocratically with water/acetonitrile/methanol (6/3/1); flow rate was set at 1 mL min^{-1} room temperature. The analyses were run on a C18 column (Supelco, 150 mm x 4.6 mm, 5 µm). The chromatograms were analysed with Autochrom-3000 system (Young Lin Instrument Co., Ltd, Anyang, Korea) version 2.0.15. AFB1 solutions ranging from 2.5 to 30 µg mL^{-1} were used for the calibration curve determination: the detection limit was 0.01 µg mL^{-1}. The percentage of AFB1removed was calculated using the equation 100 × [1 − (peak area of AFB1 in the supernatant)/(peak area of AFB1in the positive control)].

2.10. Plant growth promotion assays

To assess the *L. plantarum* ITEM 17215 ability to promote growth of wheat plants, a preliminary lab-scale test was set up. Bread wheat (*Triticum aestivum*) kernels were soaked for 24 hours in either (i) sterile distilled water, (ii) non inoculated MRS broth, or (iii) sterile distilled water and 10 mL of cultural broth containing 10^7 cells of *L. plantarum* ITEM 17215, grown in MRS medium for 24 hours at 30 °C. After soaking, the kernels were placed in plastic boxes, with natural lighting and temperature conditions, and kept wet by periodically spraying them with sterile tap water. Each treatment was set up in three distinct plastic boxes for replicates. After 7 days, the height of 100 randomly selected plants per treatment was measured.

2.11. Lactate dehydrogenase gene expression

Lactate dehydrogenase gene expression was evaluated through RT-qPCR experiments. Cells were grown at 30 °C for 48 h in MRS broth, RSM and MRS and RSM supplemented with PPA (3 g L^{-1}) and Phe (1.5 g L^{-1}). RNA extraction was performed using the NucleoSpin RNA II extraction kit (Macherey–Nagel GmbH, Düren, Germany). Residual contaminating DNA was hydrolysed with

DNaseI, Rnase free kit (Thermo Fisher Scientific) at 37°C for 30 min. Subsequently, 0.2 µg of RNA was rewritten into cDNA using RevertAid First strand cDNA Synthesis Kit (Thermo Fisher Scientific) in accordance with manufacturer's instructions. Amplification, detection, and real-time analyses were performed using a Linegene 9620 Real-Time PCR (Bioer Technology, Hangzhou, China). SG qPCR Master Mix (EURx, Gdansk, Poland) was used for real-time amplification and detection of the cDNA. The nucleotide sequences of the primers used in this study for *ldh* and reference gene (*recA*) are as reported by Marco, Bongers, de Vos, & Kleerebezem (2007). Specifically: 5' TGATCCTCGTTCCGTTGATG 3' - 5' CCGATGGTTGCAGTTGAGTAAG 3' for *ldh* gene and 5' GGCAGAACAGATCAAGGAAGG 3' - 5' TATCCACTTCGGCACGCTTA 3' for *recA*. One µL of cDNA was used as a PCR template in a 15 µL reaction mixture containing 4.78 µL water, 0.72 µL primer mix (containing 0.3 µM of each primer) and 7.5 µL of the SG qPCR master mix (EurX). The following experimental run protocol was used: initial denaturation step at 95 °C for 3 min, followed by 40 cycles of denaturation at 95 °C for 30 s, annealing at 59 °C for 30 s and extension at 72 °C for 20 s. A melting curve analysis was performed to verify the identity of the PCR products. Melting points (T_m) of 76.3 and 77.3 were obtained for *ldh* and *recA* respectively. Standard curves had good linearity (R^2 =0.999) and efficiency (slopes -2.99 and -3.13 for *ldh* and *recA* respectively). The amplification of the two genes was conducted in triplicate. The relative expression ratios were calculated by the mathematical model of Pfaffl (2001), which included an efficiency correction for real-time PCR efficiency of the individual transcripts.

2.12. Phytate degradation

Strains were preliminary grown at 30 °C for 24-48 h in modified Chalmers broth without neutral red and with 1% of sodium phytate (Sigma). Five µL of the microbial suspension were spotted on modified Chalmers' agar plates without $CaCO_3$ and with 1% of hexacalcium phytate (Sigma) (Anastasio et al., 2010). The plates were incubated at 30 °C and examined after 2 days of incubation

for clearing zones around the spots. To eliminate false positive results, caused by microbial acid production, Petri plates were flooded twice with 2% (w/v) aqueous cobalt chloride solution. After 20 min of incubation at 25 °C, the cobalt chloride solution was removed and phytase activity was evaluated.

2.13. Utilization of fructo-oligosaccharides

Fermentation of carbohydrates was tested in MRS basal medium (MRS without carbohydrate); fructo-oligosaccharides (FOS - Actilight, Tereos, Lille, France) were autoclaved separately (112 °C for 30 min) and added to the sterile basal medium to obtain the final concentration of 10 g L^{-1}. Cells from 24-h MRS cultures were washed twice with sterile isotonic saline solution, resuspended in the same isotonic solution, inoculated (1%) in MRS basal medium and in MRS-FOS and incubated at 30°C for 48 h. Fermentation of FOS was evaluated by measuring the increase in absorbance at 600 nm (A_{600}).

2.14. Statistical analysis

Three independent replicates of each experiment were performed, and their results were expressed as mean values ± standard deviation. When necessary, the data were compared through one-way ANOVA, followed by Tukey's exact test ($p < 0.05$), or through a principal component analysis (PCA), performed in SPSS statistical package for Windows, v. 22.0 (IBM Corporation, Armonk, NY, USA). The dataset used for these analyses included the 25 isolates as separate rows, and 8 columns for the different parameters evaluated: the inhibitory effect against the 5 fungal pathogens used (which were used for the PCA analysis), aflatoxin binding, production of organic acids, and phytate degradation.

3. Results and discussion

3.1 Selection of highly performant L. plantarum *strains.*

Selection of highly performant *L. plantarum* strains was carried out testing different properties. As reported in Table 1, we tested characteristics related to antifungal activity and to beneficial traits.

3.1.1. Antifungal activity

A first screening of the 25 *L. plantarum* strains was performed against five fungal target strains, *Fusarium verticillioides, Aspergillus niger, A. flavus, Mucor circinelloides* and *Penicillium roqueforti.* Varying degrees of inhibition were detected against the tested moulds in the overlay method. On the basis of the inhibition zones, *L. plantarum* strains were classified into three groups with no, moderate or strong antifungal activity (Fig. 1). *P. roqueforti* FS 22 was the most resistant strain: only four strains out of 25, one from cereals and three from cheeses, were able to inhibit its growth. In contrary, *F. verticillioides* FSq1 was inhibited by all *L. plantarum* strains, with only one exception and *M. circinelloides* FSq2 by 60% of the tested strains, most of which isolated from cereals. The data regarding fungal inhibition, analysed through the PCA, showed that the strains isolated from maize were clearly distinguishable from those isolated from other substrates and very similar to one another (Fig 2). In contrast, the strains obtained from wheat, cheese, and cocoa do not clearly segregate and have a more varied antifungal effect. These data are of interest, because growth inhibition of these contaminants has not been extensively studied and to the best of our knowledge, no reports on this antifungal ability in *L. plantarum* are available in literature. *Fusarium* species are associated with cereal grains. A few LAB strains have been shown to inhibit growth and production of mycotoxins by *F. graminearum*, which is abundant in various cereal crops and processed grains. *F. verticillioides* is the most prevalent fungus associated with maize, causing

several diseases, and producing a wide range of mycotoxin that includes fusaric acid, fusarins and fumonisins (Deepa & Sreenivasa, 2017) Fumonisin B1 is considered the most toxic one; epidemiological data indicate a possible correlation between the consumption of fumonisin/*F. verticillioides* contaminated maize and the high incidence of esophageal cancer in countries where maize is a dietary staple (Oldenburg, Höppner, Ellner, & Weinert, 2017; van Rensburg et al., 2015). *M. circinelloides* is frequently isolated from food matrices (Lee et al., 2014) and it is considered one of the causal agents of the fungal infection mucormycosis (Pitt & Hocking, 2009). Moreover, most of the *L. plantarum* analysed (68%) exerted a strong inhibition against *A. flavus* FS13, while *A. niger* FS11 was inhibited by approximately 36% of the *L. plantarum* strains. Also these data are of interest, since the results of previous studies showed the inability of *L. plantarum* to inhibit *Aspergillus* growth (Cortés-Zavaleta et al., 2014; Russo et al., 2016). In particular, as reported below, selected *L. plantarum* ITEM 17215 can completely inhibit mould development when grown in co-culture, and this ability could be effective in limiting the mycotoxin production.

L. plantarum strains were further tested for their ability to bind aflatoxin B1 (AFB1). Although in cereal sector the potential of using microbial strains with mycotoxin binding abilities is considered of great value in reducing the mycotoxin exposure (Dalié et al., 2010; Shetty & Jespersen, 2006), up to now very few *L. plantarum* have been characterized for their toxin binding ability. Overall, in the present study, *L. plantarum* strains, also tested at different incubation times, moderately bound the AFB1. The 80 % of the strains showed a binding level ranging from 5 to 7.5 μg mL^{-1}, and only a single strain (SE90) bound 29 % (14.5 μg mL^{-1}). However, in the condition of the assay, *L. rhamnosus* GG, chosen as a positive control, showed a similar binding ability, in contrast to other studies in which a percentage of 70-80% was reported (El-Nezami et al., 1998; Haskard et al., 2001). This aspect needs to be further investigated.

3.1.2. Enhancement of food quality

L. plantarum strains have been tested for targeted properties linked to improvement of food quality, as phytate degradation ability and FOS utilization. As shown in Table 1, 19 of the 25 strains were able to hydrolyse calcium phytate, an important characteristic in the cereal sector. Phytate is known to chelate several essential nutrients and can negatively influence the activity of digestive enzymes by chelation of mineral cofactors or by interacting with proteins. Moreover, 23 out of the 25 tested *L. plantarum* strains were able to grow in presence of FOS, prebiotic oligosaccharides used in combination with probiotic microorganisms for the development of functional food ingredients (Rastall & Maitin, 2002). These characteristics could be exploited for a potential use of selected *L. plantarum* strains as new probiotic cultures. Further studies are underway to find other functional properties in *L. plantarum* strains, to be followed by clinical and *in vivo* studies to confirm the strains as probiotics. Finally, all tested strains showed a high acidification rate, with a pH value, after 16 h of incubation at 30°C, ranging from 3.9 to 4.2.

3.2. Characterization of L. plantarum *ITEM 17215*

The initial screening data indicate that the tested *L. plantarum* strains demonstrated a good performance, regarding either antifungal activity or beneficial properties. Among them, the strain ITEM 17215 showed the strongest inhibitory activity towards all the tested fungal strains (Fig.3), and the ability to degrade phytate and utilize FOS. Its multi-properties, exploited in a specific process or sector, as cereal conservation and fermentation, can improve, at the same time, safety and quality of the final product. For these reasons, the subsequent experiments were directed towards the study of this strain.

3.2.1. Identification and quantification of antifungal compounds

L. plantarum ITEM 17215 completely inhibited mycelial growth of *A. flavus* and *F. verticillioides* and reduced by 25% the mycelial mass of *M. circinelloides* after 9 days of

simultaneously cultivation in malt extract broth. The antifungal activity of the strain was also tested in plates of MEA supplemented with increasing concentrations of untreated CFS*:* CFS was able to inhibit the growth of *F. verticillioides* strain when supplemented at 10% (v/v). The complete inhibition of *A. flavus* and *M. circinelloides* strains was reached when CFS was supplemented at 80%.

With the aim to identify the active antifungal compounds, CFS from the strong inhibitor strain ITEM 17215 was analysed by UPLC/PDA-ESI-HR-MS/MS. *L. plantarum* ITEM 17215 produced 1,2-dihydroxybenzene, benzoic acid, p-hydroxyphenyllactic acid and PLA (Fig.4). The latter compound, already described as efficient antifungal inhibitor, was the most abundant and its concentration, as determined by HPLC, resulted equal to 99.6±4.0 mg L^{-1}. HPLC analysis also revealed the ability of the strain ITEM 17215 to produce a discreet amount of acetic acid (1.1±0.2 g L^{-1}), combined with high lactic acid yield (21.5±1.4 g L^{-1}).

In parallel, we studied the effect of organic acids on germination of the mould spores in solid media. Acetic acid and PLA were the compounds more effective for the inhibition of growth of the studied moulds. Indeed, MIC values of 2.35, 0.58, 0.29 g L^{-1} for acetic acid were obtained for *M. circinelloides*, *A. flavus* and F. *verticillioides*, respectively. MIC values for PLA were 5.0 g L^{-1} for *A. flavus*, 3.0 g L^{-1} for *M. circinelloides* and 1.5 g L^{-1} for *F. verticillioides.* Against lactic acid, the tested moulds required higher amounts of this organic acid, being M. *circinelloides* more resistant (MIC 25.0 g L^{-1}) than A. *flavus* and *F. verticillioides* (MIC 12.5 g L^{-1}). In any case, most of the MIC obtained for organic acids against the tested moulds were greater than those estimated with the producer strain. The data of the obtained MIC are in accordance with the previous observations that more metabolites produced by LAB can contribute to synergistically inhibit mould growth (Cortés-Zavaleta et al., 2014; Russo et al., 2016).

3.2.2. L. plantarum *ITEM 17215 mould inhibition activity in cereals and milk-based products*

When inoculated in micro-silos contaminated with *A. flavus*, *F. verticillioides* or *M. circinelloides*, *L. plantarum* strain ITEM 17215 was able to inhibit the growth of the moulds (Fig. 5): after 10 days of incubation the count in MEA decreased from 10^8 CFU g^{-1} in the control micro-silo inoculated with 10^4 spores g^{-1} to <10 CFU g^{-1}.

Moreover, germination tests indicated that the *L. plantarum* strain ITEM 17215 strongly induced wheat germination and promoted plant growth (Fig. 6). Seven days after the soaking treatment, wheat kernels germinated in the plastic boxes and their heights were significantly (p <0.05) different based on the treatments. In particular, non-treated controls and MRS non inoculated-treated kernels were approximately of the same height, with an average of 3.8±0.4 cm and 3.5±0.5 cm, respectively, while the kernels treated with the cultural broth were of 6.6±0.3 cm. The statistical analysis confirmed that the cultural broth treatment is significantly different from the other two treatments (p = 0.000).

Plant growth promoting effects of some rhizobacteria have been studied: the bacteria may secrete organic acids, such as succinic and lactic acids, and these acids may increase plant growth under conditions in which the populations of pathogens are reduced (Lugtenberg & Kamilova, 2009). Little data are available on LAB as plant growth promoting bacteria: they were found as endophytic components of durum wheat plant (Minervini et al., 2015) and some studies indicate their potential for biocontrol and rice growth promotion. Adachi et al. (2103) demonstrated that PLA can promote root and shoot growth in rice seedling and hypothesize that this acid could be involved in the regulation of the cell enlargement and division in synergy with other plant growth substances. Considering the reported data, it is possible to relate the superior seedling growth we observed, with the production of organic acids, particularly PLA secreted by the strain ITEM 17215. This fact needs to be further examined before proposing a potential application of the strain as a plant growth-promoting bacterium.

L. plantarum ITEM 17215 did not show evident antagonistic effects against *P. roqueforti* used as a test mould in micro-yogurt preparation. This last result was related to a poor ability of the strain to

produce PLA when grown in milk, as verified by HPLC analysis: when the strain was grown in RSM, the PLA production was not quantifiable.

3.2.3. Improvement of PLA production

As previously reported (Valerio, Di Biase, Lattanzio, & Lavermicocca, 2016), PLA production can be improved by the addition of Phe to the growth medium and/or by the addition of the precursor PPA, with lactate LDH activity responsible for the efficient conversion from PPA to PLA. In this regard, it has been suggested that differences in the LDH amino acid sequences could be responsible for the disparity in the ability of LAB strains to produce PLA (Zhang et al., 2014). When the *L. plantarum* ITEM 17215 was grown in MRS supplemented with Phe and PPA, the production of PLA increased from about 0.1g L^{-1} to 2.2 ± 0.2 g L^{-1}, 22-fold more than in MRS broth without PLA synthesis precursors. The gene expression experiments also showed that the expression of *ldh* gene was boosted with the addition of the precursors. Statistically significant difference ($p< 0.05$) was noticeable between *ldh* expression levels (relative expression ratio of 7.84) in the two different tested conditions (Fig.7).

To verify the inability of the strain to produce PLA when grown in RSM, the *ldh* expression experiments were also carried out to evaluate the different expression level in RSM and RSM supplemented with the precursors. Transcription of *ldh* gene was poorly induced in response to precursors (relative expression rate of 1.3) (Fig.7). Moreover, *ldh* gene was over-expressed only in MRS broth (15.5-fold) in comparison to RSM. The inability of the strain to produce high levels of PLA when grown in RSM deserves to be further investigated, also at the species level.

4. Conclusions

Many reports describe the ability of *L. plantarum* strains to inhibit mould growth, related to the production of PLA and other organic acids. Moreover, the potential use of selected *L. plantarum*

strains in sourdough fermentation has been described by many authors. This study aimed to further explore the potential of the species. The tested strains not only showed a good spectrum of inhibition against food spoiling moulds, but had interesting beneficial traits such as the ability to utilize FOS, prebiotic substances, that help probiotic microorganisms to grow in the human gut, and to reduce phytate, an antinutrient present in cereal sector. Phytic acid is the major storage form of phosphorous comprising 1–5% by weight in cereals (Schlemmer, Frølich, Prieto, & Grases, 2009), but represents an antinutritive factor for its ability to chelate dietary minerals, reducing their bioaccessibility and bioavailability. Several studies have shown that the fermentation process can significantly reduce phytic acid, not only by an activation of endogenous phytases but also by specific microbial biotypes able to produce extracellular phytases (Lopez et al., 200; Manini et al., 2014). Regarding mould inhibition, we described, for the first time, the ability of the *L. plantarum* strains to inhibit moulds that have not been studied yet: the food borne pathogen *M. circinelloides* and the mycotoxigenic *F. verticillioides*. Moreover, most of the analysed *L. plantarum* strongly inhibited *A. flavus*. The selected *L. plantarum* ITEM 17215 showed the highest inhibitory ability towards all the tested fungal strains. Further analyses demonstrated that the strain produced two known fungal growth inhibitors, PLA and p-hydroxyphenyllactic acid as well as lactic and acetic acids. Regarding PLA, the presence of Phe and PPA in the growth medium, further increased its production, allowing to achieve a very high concentration of PLA. The metabolites produced by the strain ITEM 17215 could also explain its ability to induce wheat germination and to promote plant growth. This aspect, not investigated yet in *L. plantarum*, could have interesting application in the agro-food sector.

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Conflicts of interest

The authors declare no conflict of interest.

Author contributions

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706 **Table 1.**

707 Antifungal activity, aflatoxin binding, acidifying activity, phytate degradation and FOS utilization by *Lactobacillus plantarum* strains.

Strains	Antifungal activity[1]					Aflatoxin B1 binding[2]	Acidifying activity[3]	Phytate degradation	FOS utilization (A_{600})	
	Mucor circinelloides	*Fusarium verticillioides*	*Aspergillus niger*	*Aspergillus flavus*	*Penicillium roqueforti*				Basal MRS medium	MRS +FOS
CR62	+	+	-	+	-	14±4.9	4.00±0.01	+	0.61±0.05	1.91±0.12
CR63	+	+	-	+	-	12±0.4	4.16±0.00	+	0.57±0.07	1.73±0.15
CR67	+	+	-	+	-	10±0.7	4.06±0.07	+	0.58±0.03	1.80±0.05
CR70	+	+/-	-	-	-	13±0.1	3.94±0.01	+	0.95±0.04	1.61±0.06
CR71	+	+	+/-	+	-	13±0.05	3.99±0.02	+	0.94±0.02	1.86±0.10
CR73	+	+/-	-	-	-	11±1.9	3.93±0.01	+	0.94±0.05	1.79±0.02
CR74	+	+	-	+/-	-	13±0.7	4.00±0.00	+	0.66±0.06	1.65±0.01
CR75	+	+	-	+	-	10±0.8	3.97±0.00	+	0.92±0.01	1.86±0.11
CR77	+	+	-	+	-	9±0.7	3.97±0.00	+	0.90±0.03	1.53±0.07
CR78	+	+/-	-	+	-	8±1.0	3.97±0.02	+	0.96±0.03	1.63±0.09
CE42	-	+/-	+	+	-	10±1.3	3.95±0.02	+	0.97±0.02	1.80±0.14
CE60	-	+	+	+/-	-	11±1.4	3.91±0.04	-	0.78±0.07	0.72±0.10
ITEM 17215	+	+	+	+	+	14±0.6	4.21±0.01	+	0.85±0.10	1.82±0.04
93	-	+	-	+	-	12±1.9	3.88±0.01	-	0.87±0.08	1.75±0.06
MD143	+/-	-	-	+/-	+	12±0.5	3.92±0.00	+	0.89±0.00	1.79±0.05
MD123	-	+/-	-	+	-	14±0.2	3.90±0.02	-	1.01±0.05	1.76±0.10
MD147	-	+	-	+	-	7±1.6	3.87±0.02	+	0.85±0.04	1.77±0.11
VS516	-	+	-	+	-	11±1.7	3.92±0.00	-	0.98±0.01	1.84±0.10
VC114	-	+/-	+	+	-	10±0.8	3.93±0.01	+	0.78±0.08	1.68±0.01
VC194	-	+	-	+	-	11±0.2	3.90±0.01	-	0.92±0.07	1.68±0.05
VC233	+	+	+	+/-	+	11±0.6	4.10±0.01	+	0.66±0.03	1.67±0.05
SE90	+/-	+/-	+	+	-	29±0.8	3.88±0.01	+	0.81±0.03	0.82±0.06
SE140	-	+	-	-	+	11±2.1	3.90±0.00	+	0.78±0.01	1.72±0.16
AC	-	+	+	+	-	15±1.1	3.87±0.05	-	0.90±0.09	1.61±0.13
LC1	+/-	+	+	+/-	-	5±0.5	3.96±0.00	+	0.85±0.07	1.76±0.06

708 [1] Strains were classified as no (-), moderate (+/-), or strong (+) inhibitors by using the overlay method.

709 [2] Expressed as percentage of binding.

710 [3] Measured as pH value after 16 h of incubation at 30°C in MRS broth

Fig. 1. Degree of mould growth inhibition calculated on the basis of the inhibition zone: A) moderate antifungal ability; B) strong antifungal ability.

Fig. 2. Graphical representation of the two major component obtained from principal component analysis carried out on the fungal inhibition results. The principal component 1, on the x axis, explains 32% of variability, while principal component 2, on the y axis, explains 21% of variability.

Fig.3. Antifungal effect of *L. plantarum* ITEM 17215 against: A) *Aspergillus niger*; B) *A. flavus*; C) *Fusarium verticillioides*; D) *Mucor circinelloides*; E) *Penicillium roqueforti*

Fig. 4. HR-MS chromatograms of potential antifungal compounds identified in CFS from the strong inhibitor strain *L. plantarum* ITEM 17215: A, HR-MS total ion current chromatogram; B, extracted ion chromatogram ([M–H+]– exact mass ± 3 ppm) (XIC) of p-hydroxyphenyllactic acid (181.0506); C, XIC of 1,2-dihydroxybenzene (109.0295); D, XIC of benzoic acid (121.0295); E, XIC of PLA (165.0557). Retention times of corresponding compounds are indicated. Identity of compounds was confirmed by HR-MS/MS (spectra not shown).

Fig. 5. Growth inhibition of *Fusarium verticillioides* by *L. plantarum* ITEM 17215. A) micro-silo inoculated with 10^4 fungal spores g^{-1}; B) micro-silo inoculated with 10^4 fungal spores g^{-1} and 10^6 CFU g^{-1} of strain ITEM 17215.

Fig.6. Effect of *L. plantarum* ITEM 17215 on wheat germination. A) wheat grains soaked in water (control); B) wheat grains soaked in water with 10 mL cultural broth of *L. plantarum* ITEM 17215.

Fig.7. Relative expression ratio of *ldh* gene in *L. plantarum* ITEM 17215, grown in MRS and RSM in presence of 3.0 g L^{-1} phenylpyruvic acid (PPA), calculated relative to its expression in control sample. Data were normalized using *recA* gene. The bars represent the standard deviations.

Fig.1

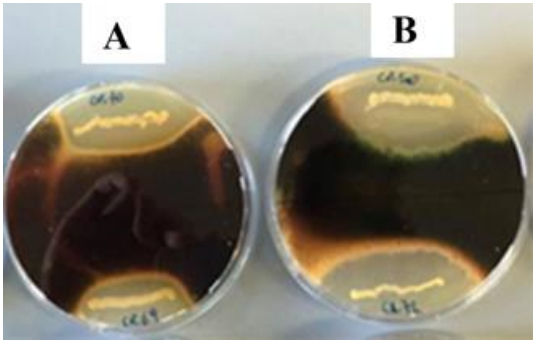


Fig. 2

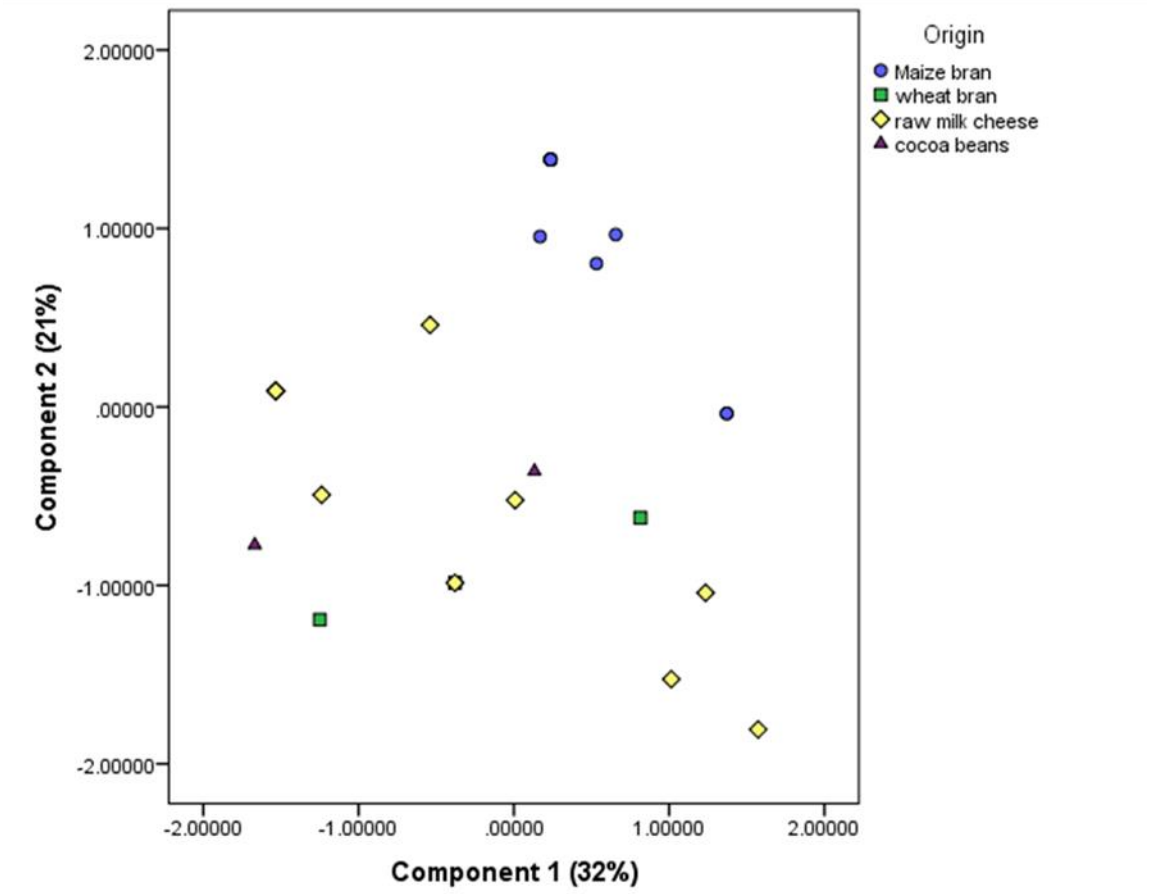
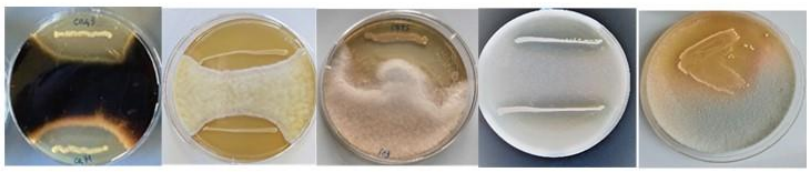

Origin
Maize bran
wheat bran
raw milk cheese
cocoa beans
2.00000
1.00000
.00000
-1.00000
-2.00000
Component 2 (21%)
-2.00000
-1.00000
.00000
1.00000
2.00000
Component 1 (32%)

Fig. 3



A B C D E

Fig. 4

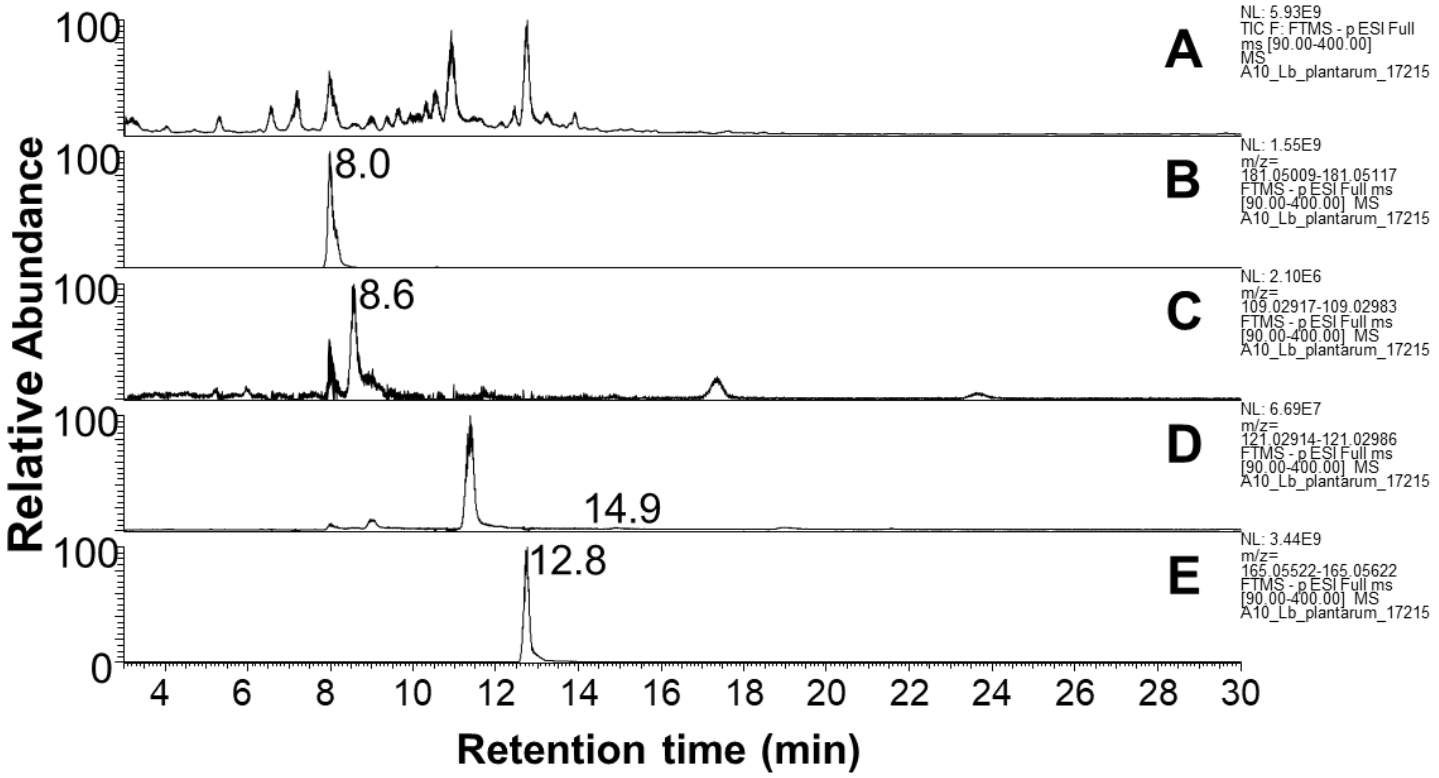

Relative Abundance
100
100
100
100
100
0
8.0
8.6
14.9
12.8
A
B
C
D
E
NL: 5.93E9
TIC F: FTMS - p ESI Full ms [90.00-400.00] MS A10_Lb_plantarum_17215
NL: 1.55E9
m/z= 181.05009-181.05117 FTMS - p ESI Full ms [90.00-400.00] MS A10_Lb_plantarum_17215
NL: 2.10E6
m/z= 109.02917-109.02983 FTMS - p ESI Full ms [90.00-400.00] MS A10_Lb_plantarum_17215
NL: 6.69E7
m/z= 121.02914-121.02986 FTMS - p ESI Full ms [90.00-400.00] MS A10_Lb_plantarum_17215
NL: 3.44E9
m/z= 165.05522-165.05622 FTMS - p ESI Full ms [90.00-400.00] MS A10_Lb_plantarum_17215
4 6 8 10 12 14 16 18 20 22 24 26 28 30
Retention time (min)

Fig. 5

Fig.6

Fig.7