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Abstract

The present work was carried out to identify lactic acid bacteria (LAB) from wheat flours and to evaluate their technological capabilities for potential incorporation in sourdough process. Six samples of baking wheat flours were collected from mills situated in different geographical regions of Tunisia and used for microbiological analysis of total mesophilic aerobic microorganisms, yeasts, moulds and LAB. A total of 45 autochthonous LAB were isolated and identified by genetic analysis of 16S-23S rRNA intergenic transcribed spacer ITS generated patterns ITS-PCR. One representative strain of each ITS-PCR pattern was subjected to partial sequencing of the 16S rRNA. Strains were identified as *Weissella cibaria*, *Lactobacillus plantarum*, *Lactobacillus brevis*, *Pediococcus pentosaceus*, *Pediococcus pentoseus*, *Pediococcus acidilactici*, *Enterococcus faecium*, *Enterococcus casseliflavus* and *Enterococcus faecalis*. Technological features including acidification, antimicrobial, amylolytic, proteolytic and antioxidant activities of six selected LAB strains were investigated for future in situ applications. Moreover, LAB were investigated for their ability to produce exopolysaccharides. All tested LAB showed good acidifying ability by decreasing significantly ($p<0.05$) the pH of flour extract below 4.0 after 24 h and below 3.0 after 72 h. *P. pentoseus* and *P. acidilactici* presented fermentation quotient FQ (ratio of lactic and acetic acids) close to the optimal range. All flour LAB isolates, demonstrated extracellular proteolytic activity. *W. cibaria* S25 had the highest radical-scavenging activity with a rate of 25.57%. *L. plantarum* S28 demonstrated the highest amylolytic activity (1386 U/mL) followed by *P. acidilactici* S16 (1086 U/mL). Although, *L. plantarum* S28 showed the highest production of exopolysaccharides (0.97 g/L). Moreover, different halo of inhibition were detected against *E. coli*, *Staphylococcus aureus*, *Aspergillus niger* and *Penicillium expansum*. This study revealed that autochthonous flour LAB exhibited interesting technological features and thus could be used in sourdough production.

Keywords: Baking flour, lactic acid bacteria, acidification, proteolytic, amylolytic, antimicrobial activity.

Introduction

Cereals are one of the most important food sources worldwide for both humans and animals. They supplies a considerable portion of the nutrients required for growth, well-being and maintenance of health (FAO 2002; FAO 2014). In the past, many populations of several cultures are directly linked to cereals and, thus are recognized by the cereals they eat. This is the case of Mediterranean people which are recognized to be "wheat people" (Alfonzo et al. 2013). Tunisia is placed among the largest wheat per capita consumers in the world with a total of 2.8 million metric tons MT per year between 2016/2017 (USDA 2016). Moreover, wheat bread is the most popular staple food consumed in Tunisia under different forms such as backer's yeast bread, Tabouna, Mlawi, Mtabga (Mamhoud et al. 2016).

However, cereal grains, including wheat, are naturally contaminated by microorganisms (yeast, moulds and bacteria) which can occur in the field during growth, harvest, post-harvest and storage (Eglezos 2010).

During milling, a small fraction of microorganisms initially concentrated in the outer layers of kernels, could contaminate the flour, leading to microbial rate increase during storage (Berghofer et al. 2003). The microflora composition and the relative species ratio on wheat grains are influenced by several factors including temperature, moisture, physical damage caused by molds and application of pesticides (Karlsson et al. 2014; Fleurat-Lessard 2017). Several works highlights that flour's wheat are dominated by fungi. Food spoilage by moulds and the occurrence of their mycotoxins constitute a potential health hazard (Čonková et al. 2006).

Among the microorganisms associated with flours, lactic acid bacteria (LAB) play an important role in preserving the balance of microbial flora and enhancing the shelf life of final products by controlling and inhibiting spoilage organisms during fermentation, due to their antimicrobial properties (Dalié et al. 2010; Cizeikiene et al. 2013).

LAB are commonly present in various habitats, including milk and dairy products, vegetables, meat and cereal products, where fermentation can take place (Giraffa 2014) and they constitute the majority of the rate of the commercial starter cultures (Corsetti and Settanni 2007; De Vuyst et al. 2009; Guyot 2010). LAB have been isolated, identified and characterized from cereals (Mamhoud et al. 2016) or mature sourdoughs (Digaitiene et al. 2012) or fermented dough (Mhir et al. 2007).

However, few studies were focused on the characterization of LAB isolated from wheat flours (Corsetti et al. 2007; Alfonzo et al. 2013). To our knowledge, there are no previous studies focused on the characterization of lactic acid bacteria isolated from Tunisian wheat flours.

Despite the availability of essential nutrients for LAB growth, the investigation of flour's LAB content remains difficult and critique because of the low water content (Alfonzo et al. 2013). Indeed, within flours, several LAB species are found in a dormant state (Corsetti et al. 2007). Some studies showed that *Lactobacillus* and *Enterococcus* were the most abundant in Italian flours (Corsetti et al. 2007; Robert et al. 2009). The aims of this study were to: (i) enumerate total mesophilic aerobic microorganisms, yeasts, fungi and LAB in flour samples; (ii) isolate, identify and characterize LAB naturally occurring on Tunisian wheat flour; (iii) and evaluate the technological capabilities of LAB by examining their acidifying ability in a flour extract system, proteolytic, amylolytic, antibacterial and antifungal activities for a further incorporation as starter in sourdough formulation.

Materials and Methods

Sampling and microbiological analyses

Six samples of baking wheat flours were collected from mills situated in different geographical regions of Tunisia (Nabeul, Tunis, Sousse, Djerba and Sfax), and used for microbiological analysis of total mesophilic aerobic microorganisms, yeasts, moulds and Lactic acid bacteria (LAB) (Table 1). Ten grams of flour were homogenized with 90 mL of sterile peptone water solution (peptone 0.1% and NaCl 0.85%) (Minervini et al. 2015). Total mesophilic aerobic microorganisms, yeasts and moulds were enumerated using the PCA (Plate Count Agar) and SD (Sabouraud Dextrose) agar media with chloramphenicol 5%, respectively (Minervini et al. 2015). Lactic acid bacteria (LAB) were enumerated using MRS medium (Man, Rogosa, Sharpe agar) supplemented with 0.01% of cycloheximide (MP Biomedicals) to avoid fungal growth and 0.0025% of bromocresol green (MP Biomedicals) to indicate acidification of the medium (yellow turn) by LAB.

Isolation of lactic acid bacteria

The isolation of lactic acid bacteria was carried by enriching 10 g of each sample into 90 mL of modified-MRS (mMRS) broth (1% maltose, 1% lactose and 10% yeast extract). Cultures were incubated at 30°C for 3 days. Then 1 mL of each resulting culture was diluted using decimal dilutions and plated in MRS agar supplemented with 0.0025% of bromocresol green (MP Biomedicals) and 0.01% cycloheximide (MP Biomedicals) and incubated under anaerobic conditions at 30 °C for 48-72 h. Acid-producing bacteria characterized by a yellow zone around each colony were picked and purified on MRS agar. Gram-positive and catalase negative isolates were restreaked and cultivated into the same agar medium. The pure cultures were maintained in 20% of glycerol (v/v) and conserved at -80 °C.

DNA extraction, characterization and molecular identification of lactic acid bacteria

Genomic DNA was extracted according to the method described by Wilson (2001) using CTAB/NaCl method. Bacterial strains were characterized genotypically by profile analysis of the 16S-23S rDNA Internal Transcribed Spacer (ITS) region using universal primers (s-d-bact-1494a-20 and s-d-bact-0035-a-15) (Daffonchio et al. 1998). Bacteria strains presenting the same band patterns were clustered in the same ITS-haplotype. One or two representative strains from each cluster have been selected for 16S rDNA gene PCR amplification which was performed using universal primers (s-d-bact-0008-a-S-20 and s-d-bact-1495-a-A20) according to Daffonchio et al. (1998). The two PCR were performed on a thermocycler (BioRad) using this program: 94 °C for 3 min, 35 cycles of 94 °C (45s), 55 °C (1 min), 72°C for 2 min and a final cycle at 72 °C for 10 min. PCR products were separated by electrophoresis through 1.5% (w/v) agarose gel containing ethidium bromide (0.5 mg/mL).

The 16S rDNA PCR amplicons were purified with Exonuclease-I and Shrimp Alkaline Phosphatase (Exo-Sap, Fermentas, Life Sciences) based on the manufacturer's standard protocol. DNA sequencing was performed in an automated capillary DNA sequencer (Applied Biosystems 3130XL) using a Big Dye Terminator cycle sequencing Kit V3.1 (Applied Biosystems). Sequence identification to the closet relative taxa of the strains was achieved using BLAST analysis tool (Tamura et al. 2011) in the GenBank DNA database (www.ncbi.nih.gov). Phylogenetic analysis of 16S rRNA gene sequences was conducted with MEGA-6 software (Tamura et al. 2011). Phylogenetic tree was constructed by using neighbor-joining method (Saitou and Nei 1987).

Technological characterization of LAB

Acidification activity

To evaluate the acidification activity of isolated LAB, a sterile flour extract (SFE) was prepared according to the method described by Alfonzo et al (2013): 200 g of flour was suspended in distilled water (1 L) and sterilized at 121 °C for 20 min. After precipitation, the flour was removed. The supernatant was used as liquid broth in subsequent experiments. Overnight LAB cultures on MRS broth were centrifuged (5000 x g for 5 min and washed 3 times with sterile distilled water. LAB cells were inoculated in 50 mL of the solution SFE with 1% (v/v) of bacterial suspension at 10^9 CFU/mL and incubated at 30 °C. The pH measurements were taken initially, after 24 h, 48 h and 72 h of inoculation**.**

Strains that rapidly and greatly decreased SFE pH, were analyzed for their ability to produce lactic and acetic acids, following 24, 48 and 72h of fermentation. Each resultant sample of acidified SFE (aSFE), was mixed with 1/10 mixture of ethyl-butyric acid 2 mg mL (internal standard), filtered through 0.2-µm filters and stored at -80 °C until analysis. A system composed of a 7020A gas chromatograph (Agilent Technologies Inc., Palo Alto, CA) connected to a mass spectrometry (MS) 5975N detector (Agilent) was used to quantify the short chain fatty acids SCFA. Data were collected with Enhanced ChemStation G1701DA software (Agilent). 1 µl were directly injected into the gas chromatograph equipped with a ZB-WAX capillary column (30-mm length by 250 µm internal diameter, with a 0.25 µm film thickness; Agilent) using H_2 as the gas carrier, with a constant flow rate of 1.5 mL/min. The temperature of the injector was kept at 220 °C, and the injection was performed in split less mode. Chromatographic conditions were as follows: an initial oven temperature of 50 °C, 5 °C/min up to 180 °C, 1 min at 180 °C, and 20 °C/min up to 220 °C for cleaning the column. The column was directly connected to the MS detector, and the electron impact energy was set to 70 eV. The data collected were in the range of 25 to 250 atomic mass units (3.25 scans/s). The organic acids were identified by comparing their mass spectra with those held in the NSIT MS Data Center and 2HP-Wiley 138 library (Agilent) and by comparing their retention times with those of the corresponding standards (Volatile Free fatty acid mixture CRM46975) purchased from Sigma. The peaks were quantified as the relative total ionic count abundance with respect to the internal standard. The concentration estimated in mM of each acid was calculated using linear regression equations ($R^2 \geq 0.99$) from the corresponding curves of standards obtained with eight different concentrations.

Proteolytic activity

Extracellular proteolytic activity of LAB strains inoculated for 72 h in SFE, was measured according to the method described by Miralles et al (1996) using Azocasein as substrate. Absorbance was measured at 440 nm against a blank containing only the Azocasein. Proteolytic activity was expressed as $\Delta DO_{440} \times h^{-1} \times mg^{-1}$ dry weight.

Antioxidant activity

The antioxidant activity was measured using DPPH according to the method described by Lin and Chang (2000). LAB were harvested by centrifugation at 4400 x g for 10 min after culture of 18 h incubation at 37 °C. The test was performed on the intact bacterial cells. Cells were washed three times with phosphate buffer solution (PBS: 0.85% NaCl, 2.68 mM KCl, 10 mM Na_2HPO_4, and 1.76 mM KH_2PO_4, pH 7.4) and resuspended in the same buffer. The sample (0.8 mL) was reacted with 1 mL DPPH solution (0.2 mM in 0.5% ethanol). The control was

prepared by using ultra-pure water. The tubes were incubated for 30 min in the dark. Absorbance was measured by spectrophotometer (Thermo Scientific Multiskan GO) at 517 nm in triplicate. The results are expressed as a percentage of the anti-radical activity:

$$\text{Antioxidant activity (\%)} = [\frac{A_{517control} - A_{517sample}}{A_{517\ control}}] \text{ x } 100$$

Amylolytic activity

The amylolytic activity of LAB was measured using the starch-iodine method, described by Bartkiene et al (2013).To evaluate this activity, the sterile flour extract SFE was enriched with glucose 0.15% in order de enhance the bacterial growth. The absorbance was measured at 670 nm using a spectrophotometer (Thermo Scientific Multiskan GO). One unit of α-amylase activity (1 AU) was defined as an amount of enzyme that catalyzes 1 g soluble starch hydrolysis to dextrins in 10 min at 30 °C temperature.

Antimicrobial activity

The antimicrobial activities of LAB were determined against pathogenic bacterial and fungal strains. *Enterococcus faecalis* ATCC 29212, *Escherichia coli* DH5α, *Pseudomonas aeruginosa* ATCC 27853 and *Staphylococcus aureus* ATCC 25923 were used as indicator strains. These pathogens were grown in Brain heart infusion (BHI) broth at 37 °C. The inhibitory activity was evaluated with spots method. The colonies of LAB were grown in MRS medium incubated for 24 h at 30 °C. The indicator strains were inoculated at 10^5 CFU/mL in 5 mL of soft agar (containing 0.75% agar) and poured into petri dishes which were then incubated anaerobically at 37 °C for 24 h. Antimicrobial activity was expressed as inhibition diameter zones in millimeter (mm) against the pathological strains. The zone of inhibition was divided as follows: strong inhibitor ($d \geq 15$ mm), medium inhibitor ($11 \leq d < 15$ mm), no significant inhibitory effect ($d < 11$ mm).

The antifungal activity of LAB was evaluated against two fungi *Aspergillus niger* and *Penicillium expansum* according to the method described by Whipps (1987). LAB were inoculated on the modified MRS agar (without ammonium citrate and sodium acetate), 2 cm from the edge line of the petri dish and then grown at 30 °C for 48 h. A piece of fungus was placed in the center of the petri dish and then incubated at 25 °C for 2 to 3 days. Control plates were inoculated with fungi only. The antifungal activity of LAB was expressed according to the following formula:

$$\text{Antifungal activity (\%)} = [\frac{R2 - R1}{R1}] * 100$$

R1 is the radial distance developed by fungi in the direction of antagonism; R2 is the radial distance developed by the fungi.

Production of exopolysaccharides

The measurement of the amount of exopolysaccharides was done by the colorimetric test developed by Dubois et al (1956).The supernatant (0.4 mL) of an overnight culture of LAB strain was mixed with 0.2 mL of phenol solution (5%) and 0.5 mL of concentrated sulfuric acid. After incubation in the dark for 30 minutes, the absorbance was measured at 490 nm against a blank without LAB strain.

Statistical analysis

All experiments were repeated three times and illustrated as the mean values ± standard deviations. Statistical analyses were performed using the IBM SPSS Statistics software version 23.0. The data were analyzed using one-way analysis of variance (ANOVA), followed by the Duncan's test with the significance level set at $p<0.05$ to establish the significance of differences between the samples.

Results and discussion

Microflora of wheat flour

Examination of the microbiological quality of wheat flour samples was performed in order to gain a common view about the hygienic quality and microbiological load. The microbial content of flours is composed of mesophilic aerobic bacteria, yeasts, moulds, and lactic acid bacteria. The results of microbiological analysis showed that yeasts constituted the major microbiota of the flour samples (Table 1). Yeast counts ranged from 0.30 to 2.57 Log CFU/g, whereas presumptive LAB counts varied from 0.17 to 1.68 Log CFU/g. The mean mesophilic aerobic bacteria counts in all flour samples studied were 1.53 to 2.11 Log CFU/g. All samples were below the maximum acceptable limits of the Codex Alimentarius (FAO 1995b). According to Minervini et al. (2015), LAB contaminating flour is strongly affected by the endophytic microbiota of cereals, mainly by the plant organs, the cultivars, and the phenological growth stages. A number of plant-associated microorganisms infect grains, and so flours, touching the whole quality of leavened baked goods (De Vuyst et al. 2009; Gobbetti et al. 2014).

Isolation and identification of lactic acid bacteria

LAB were isolated from Tunisian wheat flours collected from six different regions (Table 1). Upon enrichment in MRS broth 113 LAB isolates were initially selected based on their ability to produce acid by the presence of yellow halo surrounding the colonies on MRS-bromocresol green plates and purified. A total of 45 isolates Gram-positive and catalase-negative rods and cocci were kept on MRS agar for further identification.

The isolates were subjected to ITS-PCR amplification analysis and 16S-PCR sequencing. In fact, diverse studies have previously described the efficiency of ITS for inter- and intra-differentiation at the genus/ species level (Gürtler and Stanisich 1996) due to the high variability of these spacers. The ITS-PCR amplification generated different patterns (bands ranging from 50 to 1000 base pairs). Comparing the generated patterns of PCR products obtained from the studied isolates, we distinguished 09 ITS fingerprints. One representative of each ITS-PCR pattern were subjected to partial sequencing of the 16S rRNA. LAB isolates were identified at species level by 16S rRNA gene sequencing which is generally regarded as a more reliable solution for the classification and identification of LAB (Ehrmann and Vogel 2005). According to the comparison of 16S rDNA sequences with those available in GenBank, all isolates were related to LAB species with sequence homology >97%. Phylogenetic tree of LAB isolated from wheat flours was constructed based on the 16S rDNA sequences from evolutionary distances by the neighbor-joining method (Figure 1). LAB identification showed several consistent recognized species that are affiliated to four lactic genotypic groups (Figure 1). LAB strains were identified to: *Weissella cibaria* (05 strains), *Lactobacillus plantarum* (08 strains), *Lactobacillus brevis* (04 strains), *Pediococcus pentosaceus* (10 strains), *Pediococcus pentoseus* (04 strains), *Pediococcus acidilactici* (04 strains),

Enterococcus faecium (05 strains), *Enterococcus casseliflavus* (03 strains) and *Enterococcus faecalis* (02 strains).

In our studies, several LAB species found were reported to be associated with bread production, such as wheat grains and flours (Corsetti et al. 2007). In addition, some of LAB species isolated in our wheat flours are naturally found in mature sourdoughs such as *L. plantarum* (Corsetti and Settanni 2007; Alfonzo et al. 2013) and *W. cibaria* (Alfonzo et al. 2013). We noted that *Pediococcus* genus was presented by *P. pentosaceus* (followed by *P. pentoseus* and *P. acidilactici*) as the most commonly isolated bacterium from wheat flours. *P. acidilactici* is emerging as a potential probiotic in animal and human (Guerra et al. 2007).

The dominant species of *Lactobacillus* found from wheat flours was *L. plantarum* followed by *L. brevis*. This finding reinforced the concept that *L. plantarum* has a strong ecological or metabolic adaptability to different habitats (Alfonzo et al. 2013; Minervini et al. 2015). However, these two *Lactobacillus* species were reported to dominate raw and fermented vegetables (Rhee et al. 2011). Similar findings were noted for *P. pentosaceus* (Yang and Chang 2010; Swain et al. 2014). Together with *L. plantarum* and *W. cibaria* are generally isolated from plants, wheat grains and fermented wheat products (sourdough) (Corsetti et al. 2007; Trias et al. 2008; Alfonzo et al. 2013). For *Enterococcus* genus, *En. faecium* was the most commonly species presented followed by *En. casseliflavus* and *En. faecalis*. In fact, *enterococci* are normal inhabitants of gastrointestinal tract (Giraffa 2002). The isolated *Enterococcus* strains were discarded from further studies to avoid risks for antibiotic resistance and virulence gene dissemination which contribute to the pathogenesis of virulent bacteria (Bortolaia et al. 2016). *Weissella* genus was represented at low occurrence by only *W. cibaria* species. Recording to our findings, there was no correlation between LAB species and the geographical location of the wheat flour samples.

Assessment of the technological properties of LAB isolates

Acidification activity

In order to select lactic bacteria with technological characteristics relevant for sourdough process, 06 selected representative LAB species from wheat flour samples were investigated for the acidification capacity to decrease pH of sterile flour extract SFE. The results of the acidification activity are shown in Figure 2. We note that all tested LAB strains presented good acidifying abilities. They were able to decrease the SFE pH significantly ($p<0.05$) below 4.0 after 24 h. At 72 h, almost all tested LAB strains acidified the medium below pH 3.0. Except *P. acidilactici* S16, the remained strains were fast acidifiers revealing a ΔpH (difference between pH before and after inoculation of SFE by strains) around 2.8 after 24h and a final pH (after 72h) ranging between 2.0 and 3.0. The acidifying ability of the majority of the identified LAB strains after 24 h, 48 h and 72 h incubation were similar. Compared to initial pH of incubated culture, *Lactobacillus plantarum* S28 showed the rapid acidification ability ΔpH to decrease pH significantly ($p<0.05$) of the flour extract broth at 24 h (4.84 ± 0.2). This finding result concurred with previous studies of Ventimiglia et al. (2015) which reported that *L. plantarum* is the highest acid-producing species of LAB group. It causes rapid acidification of the raw material through the production of organic acids, mostly lactic acid. Besides, it is an abundant food related species reported to be common as well for sourdough environments (Corsetti and Settanni 2007). Accordingly, their fast lowering of the pH ability improves safety and organoleptic properties of fermented food products (Hansen 2002).

The concentration of lactic and acetic acids in SFE was carried out on four strains (*P. acidilactici*, *P. pentoseus, L. plantarum* and *L. brevis*), that displayed interesting technological properties, after 24, 48 and 72 h of fermentation (Figure 3). Lactic acid concentration increased after 48 h for all LAB strains. It reached 5.73 - 8.36 mg/g. After 72 h, the content increased significantly ($p<0.05$) and reached 7.77 mg/g for *P. acidilactici* and 7.06 mg/g for *P. pentoseus*. However, we noted a significant ($p<0.05$) decline of lactic acid concentration after 72h for *L. plantarum* (6.18 mg/g) and *L. brevis* (6.79 mg/g). Acetic acid production ranged from 0 to 3.87 mg/g after 24 h, and 3.74 to 5.95 mg/g after 72 h. In fact, acetic acid can occur via the citrate metabolism (Zalán et al. 2010) or the degradation of lactic acid produced (Oude Elferink et al. 2001) which may justify the reduction of lactic acid concentration in SFE extract of *L. plantarum* and *L. brevis*

The strains displayed a fermentation quotient (FQ: molar ratio between lactic and acetic acids) varied from 0.84 to 2.38, which is considered to affect the organoleptic features (aroma and texture) and to prevent ripeness and fungi spoilage of final products and so to guarantee longer shelf life (Gobbetti et al. 2000; Datta and Henry 2006). *P. pentoseus* S14 and *P. acidilactici* S16 presented FQ close to the optimal range of 2.0– 2.7 suggested by Hammes and Gänzle (1998).

Proteolytic activity

The proteolytic activity of tested LAB was determined by their inoculation in sterile flour extract SFE. The extracellular proteolytic activity assessed by azocasein degradation was detected in all flour LAB strains (Figure 4). *P. pentoseus* S14 and *L. brevis* S12 exhibited significantly ($p<0.05$) highest proteolytic activity (1.51 ± 0.18 and 1.41 ± 0.14, respectively). The extracellular protease activity is known to improve organoleptic features of leavened baked goods by generating small peptides and free amino acids as precursors for flavor development (Cagno et al. 2002; Rizzello et al. 2014). Moreover, the extracellular protease activity is important for the rheology and staleness of breads (Corsetti et al. 2000). Moreover, proteolysis generated small peptides which are important for rapid microbial growth and acidification during fermentation (Cagno et al. 2002). In addition, certain LAB strains are further known to be able to release bioactive peptides from proteins, which are thought to have a role in promoting health (Leroy et al. 2006). This finding revealed the potential use of these LAB in bread making.

Antioxidant activity

The DPPH scavenging activity of the six LAB strains from flour samples are shown in Figure 5. The results showed DPPH scavenging activity ranging between 4.39 to 25.57%. Among the tested strains, *W. cibaria* S25 had significantly ($p<0.05$) the highest radical-scavenging activity with a rate of 25.57%, followed by *L. brevis* S12 with 24.81% and *P. pentosaceus* S30 with 21.49%. Several authors have reported that the fermentation by lactic acid bacteria with antioxidant activity is considered as one of the most important tool suitable to enhance the functional and the organoleptic potential of several fermented cereal flours (Coda et al. 2012; Rizzello et al. 2013; Curiel et al. 2015).

Amylolytic activity

The biodiversity of amylolytic lactobacilli, ubiquitously used in cereals processing, is quite limited (Reddy et al 2008). In this work, the *in-vitro* amylolytic activity of the tested LAB was evaluated by starch-iodine method. According to the results presented in Figure 4, all tested LAB are characterized by starch hydrolysis. They

showed amylase activity ranging from 658± 13 to 1386± 4 U/mL (for 1 g of starch hydrolyzed (SH)) which are equivalent to 6.58 to 13.86 U/ml (for 0.01 g of SH). *L. plantarum* S28 demonstrated significantly (p<0.05) the highest amylolytic activity (1386 ± 4 U/mL) followed by *P. acidilactici* S16 (1086 ± 6 U/mL) and *P. pentoseus* S14 (1046 ± 9 U/mL). These results obtained for the amylolytic activity are in line with previous reports (Petrova et al. 2010; Amapu et al. 2016). Production of amylase has been described primordially for *L. plantarum* (Giraud et al. 1994). Besides, the amylolytic activity recorded for tested LAB strains was equivalent to 8 - 11.5 U/ml reported for amylolytic strains (Petrova et al. 2010). In fact, high amylolytic activity was reported for *L. plantarum, P. acidilactici* and *L. brevis* which are predominant amylase producing LAB in wet milled cereals (Amapu et al. 2016). Besides, Sanni et al. (2002) described amylolytic strains of *L. plantarum* in several traditional amylaceous fermented foods. The potential LAB strain with amylolytic activity could be used as one of the factors to improve the fermentation rate, reduction of dough viscosity with resultant improvements in the volume and texture of the bread for food industries (making of high density gruels, baking, brewing) (Fossi and Tavea 2013). Ray and Montet (2016) reported that they are also employed in preparing high energy density (ED) cereal-based foods for improving dietary starch utilization in infants and small children. On the basis of their technological potentials, the strains *L. plantarum* S28, *P. acidilactici* S16, *P. pentoseus* S14, *W. cibaria* S25 and *L. brevis* S12 isolated from wheat flours retains better starch degradation ability. It may developed into starter cultures (sourdoughs) in the making of fermented cereal foods, including bread, to contribute to the enzymatic pool and yield products with a higher fermentable sugar content (Amapu et al. 2016; Hattingh et al. 2015). The hypothesis that the amylolytic activity of strains studied are cell wall bound or/and produced in cell-free supernatants needs to be verified.

Exopolysaccharide production

The cell-free supernatants of selected LAB strains were examined for the amounts of exopolysaccharide (EPS) yields using phenol-sulfuric acid method. EPS production of each strain was presented in Figure 6. EPS amount produced by selected LAB isolates ranged from 0.172 to 0.970 g/L. Figure 6 obviously demonstrates that the strains *P. acidilactici* S16 and *L. plantarum* S28 showed significantly (p<0.05) highest EPS production (0.97 and 0.636 g/L, respectively). Several health benefits have been attributed to exopolysaccharides EPS from LAB which are used to improve the textural properties of fermented foods (Crescenzi 1995; Ruas-Madiedo et al. 2008). These strains can be used in sourdough and may have applications in bread making.

Antibacterial activity

In order to select bacterial strains with antibacterial activity, selected LAB were assessed against four pathogens (*Enterococcus faecalis*, *Escherichia coli, Pseudomonas aeruginosa* and *Staphylococcus aureus*) as indicator strains. Table 2 gives the results for antibacterial activity of the LAB isolates in terms of inhibition diameter around the spot. All of LAB strains presented aptitude to inhibit the growth of tested pathogens with different percentages. The antibacterial activity was recorded as strong growth inhibition against *Pseudomonas aeruginosa* for *L. brevis* S12 (15 mm), *P. pentoseus* S14 (16 mm), *W. cibaria* S25 (17 mm) and mostly for *P. pentosaceus* S30 (18 mm). Except *P. acidilactici* S16, the entire tested LAB showed a significant (p<0.05) moderate antagonistic activity towards *E. coli* and *St. aureus*. *L. brevis* S12 and *P. pentoseus* S14 presented high values of diameter of growth inhibition against *St. aureus* (14.5 mm) and *E. coli* (12 mm), respectively. Only *L.*

brevis S12 and *P. pentosaceus* S30 presented significantly (p<0.05) a medium growth inhibition activity against *En.faecalis* with an average of 11 mm. In our case, we observe that the inhibition of microbial growth by the six LAB is due to pH variation on culture media and might be a result of fermentative compounds accumulation. LAB are known to produce a range of metabolic end compounds that are able of interfering with the growth of certain undesirable microbes in food systems (Vandenbergh 1993; Alvarado et al. 2006). In fact, most of the inhibitory activity exhibited by LAB strains was attributed to pH reduction by organic acids. Besides, it is suggested that acid products interfere with permeability of plasmic membrane and raise its diffusion leading to stop metabolic activities and so to the inhibition effect of sensitive microorganisms (Piard and Desmazeaud 1991). So, these findings led to suggest that sourdough and bread produced with these tested LAB strains, showing consistent ability to retard the growth of both pathogen moulds and bacteria species, thus have the potential to improve the shelf-life of wheat bread.

Antifungal activity

All the studied lactic acid bacteria strains, isolated from different wheat flour samples, were assessed for inhibitory activity against two common post-harvest fungus *Aspergillus niger* and *Penicillium expansum* using a dual culture method. Varying degrees of inhibition were detected against the two moulds *in-vitro* (Figure 7). All LAB strains exhibited significant (p<0.05) inhibitory effects towards the tested fungi. The growth of *Penicillium expansum* was strongly inhibited by *P. pentoseus* S14 with an inhibitory rate of 84%. While growth of *Aspergillus niger* was moderately (p<0.05) inhibited by all the tested LAB isolates from 16.7% to 36.1% of inhibition. Almost all strains, especially *L. plantarum* S28 and *P. acidilactici* S16, showed radial (p<0.05) growth reductions against *P. expansum* and *A. niger* by an average of 36 % and 25%, respectively. *Penicillium expansum* was the most sensitive strain in dual-culture method. Dal Bello et al. (2007) and Djossou et al. (2011) reported that *L. plantarum* isolated from sourdough and plant materials is known by its antifungal activity. This study revealed that Tunisian wheat flours included autochthonous and selected LAB of interesting technological features relevant to sourdough production. In fact, the obtained results might be helpful to use a mixed starter culture including *L. plantarum, L. brevis, P. acidilactici and P. pentoseus* for producing sourdough bread.

Acknowledgments

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609 **Table 1.** Microflora in flour samples collected from mills located throughout Tunisia (Log CFU/g).

Sample	Company	Geographical origin	Mesophilic aerobic bacteria	Yeasts	Moulds	Lactic acid bacteria
E1	Grands Moulins de Nabeul	Nabeul	1.89 ± 0.17^{b}	2.51 ± 0.11^{a}	$<1^{*}$	1.68 ± 0.03^{a}
E5	Epis de Carthage	Sousse	1.72 ± 0.11^{b}	2.27 ± 0.20^{a}	$<1^{*}$	1.16 ± 0.15^{b}
E6	RANDA	Tunis	1.89 ± 0.14^{b}	2.57 ± 0.17^{a}	$<1^{*}$	1.67 ± 0.04^{a}
E7	Couscousseries du sud	Sfax	2.11 ± 0.24^{a}	2.41 ± 0.21^{a}	$<1^{*}$	1.21 ± 0.07^{b}
E8	Epi d'or	Sousse	1.53 ± 0.12^{c}	0.30 ± 0.04^{c}	1.35 ± 0.12^{a}	0.17 ± 0.04^{c}
E11	AZIZA	Djerba	2.04 ± 0.21^{a}	1.27 ± 0.12^{b}	0.39 ± 0.07^{b}	0.30 ± 0.05^{c}

610 Each value represents the mean value standard deviation (SD) from two trials; a, b and c, represent significant (p<0.05)
611 differences in the same column; *: Counts<1.
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Table 2. Inhibitory diameter (mm) of neutralized cell-free supernatant of six selected LAB flour isolates.

	Enterococcus faecalis ATCC 29212	***Pseudomonas aeruginosa*** ATCC 27853	***Escherichia coli*** DH5α	***Staphylococcus aureus*** ATCC 25923
***Pediococcus acidilactici* S16**	8.5 ± 0.71^{a}	11.5 ± 0.71^{b}	8 ± 0^{a}	10.75 ± 0.35^{a}
***Pediococcus pentoseus* S14**	10 ± 0^{b}	16 ± 0^{a}	12 ± 1.41^{b}	11.75 ± 1.06^{b}
***Weissella cibaria* S25**	8 ± 0^{a}	17 ± 0^{a}	11 ± 0.71^{b}	12 ± 0^{b}
***Lactobacillus brevis* S12**	11.5 ± 0.71^{b}	15 ± 0^{a}	11 ± 0^{b}	14.5 ± 0.71^{c}
***Lactobacillus plantarum* S28**	9 ± 1.41^{a}	13 ± 0^{a}	11 ± 1.41^{b}	12.5 ± 0^{b}
***Pediococcus pentosaceus* S30**	11 ± 0^{b}	18 ± 0^{a}	11.5 ± 0.71^{b}	11.75 ± 0.35^{b}

Numbers indicated the diameter of the inhibition zone in mm; Each value represents the mean value standard deviation (SD) from two trials; a, b and c, represent significant ($p<0.05$) differences in the same column**.**

Figure captions

Fig. 1 Dendrogram obtained by comparing 16S rDNA sequences of the isolates from Tunisian wheat flours, based on 16S rDNA partial sequences, using the neighbor-joining method. Bootstrap values for a total of 1000 replicates are shown at the nodes of the tree, using MEGA-6. The scale bar corresponds to 0.01 units of the number of base substitutions per site.

Fig. 2 Kinetics of acidification of wheat flour LAB. Histograms are in the order of increasing pH after 24h, 48h and 72 h.

Fig. 3 Organic acids content of wheat flour LAB (mg/g) after 24h, 48h and 72 h.

Fig. 4 Proteolytic (grey) and amylolytic (black) activities of wheat flour LAB.

Fig. 5 Antioxidant activity (%) of wheat flour LAB.

Fig. 6 Exopolysaccharide production (g/L) of wheat flour LAB.

Fig. 7 Antifungal inhibition (%) of wheat flour LAB against *Aspergillus niger* and *Penicillium expansum.*

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724 **Fig. 1**
725

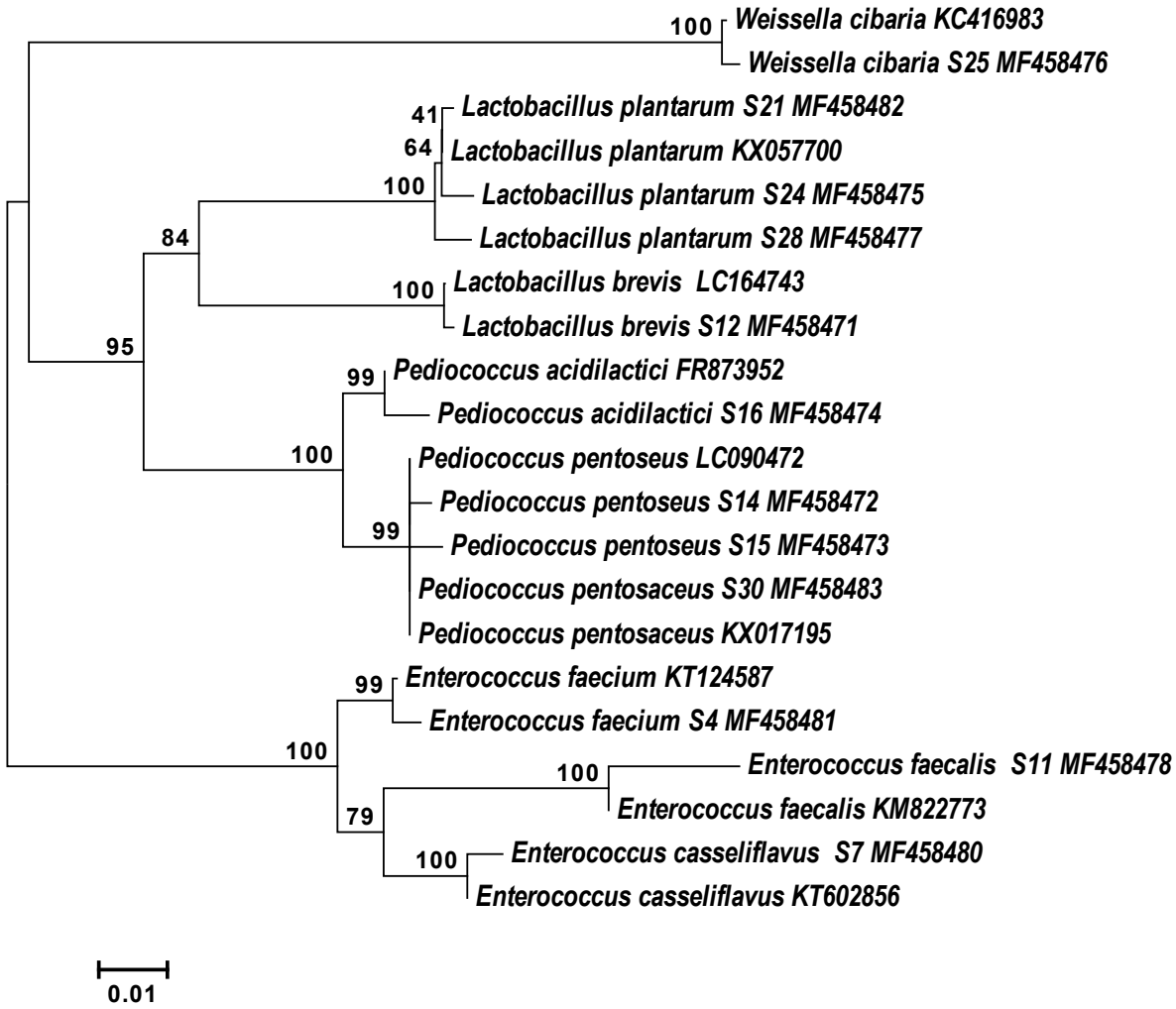

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Fig. 2

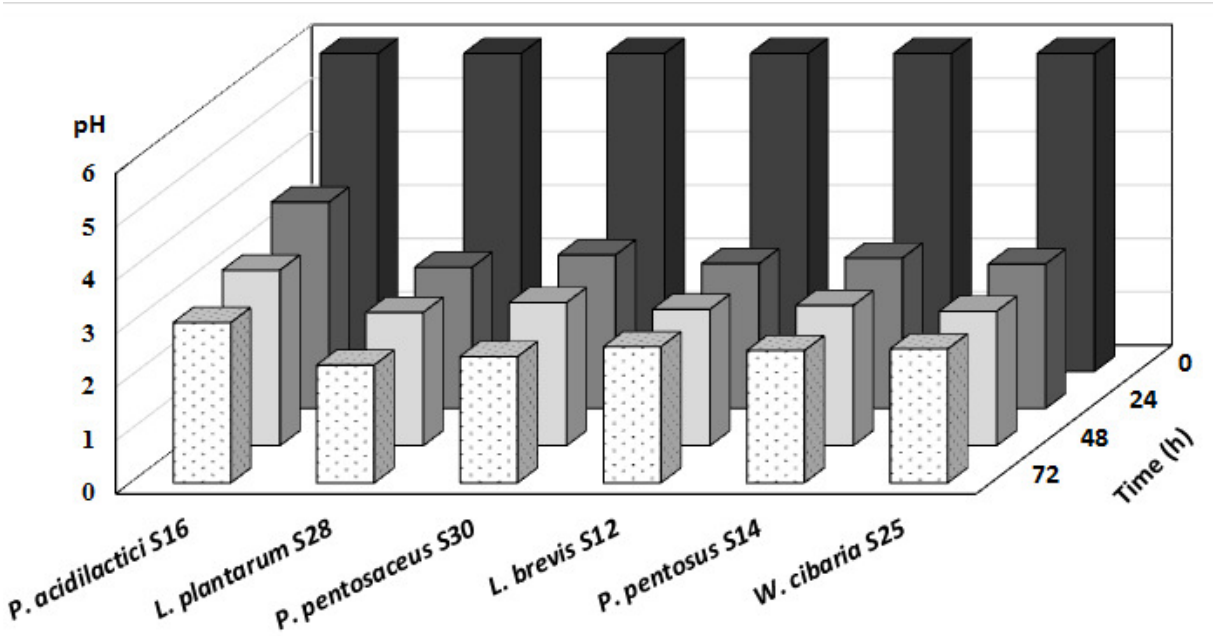

pH
6
5
4
3
2
1
0
0
24
48
72
Time (h)
P. acidilactici S16
L. plantarum S28
P. pentosaceus S30
L. brevis S12
P. pentosus S14
W. cibaria S25

Fig. 3

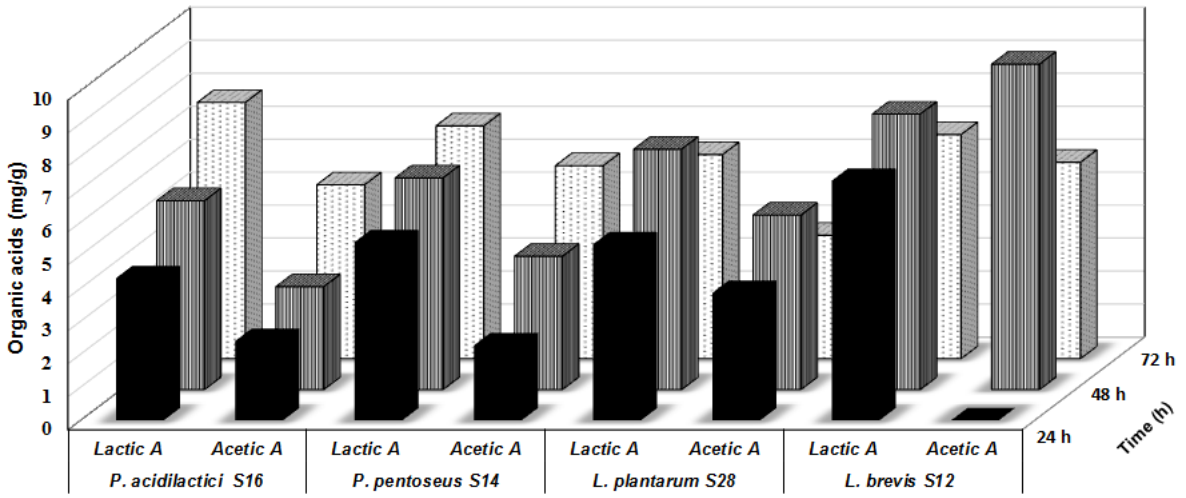

Organic acids (mg/g)
0
1
2
3
4
5
6
7
8
9
10
Lactic A
Acetic A
P. acidilactici S16
P. pentoseus S14
L. plantarum S28
L. brevis S12
24 h
48 h
72 h
Time (h)

Fig. 4

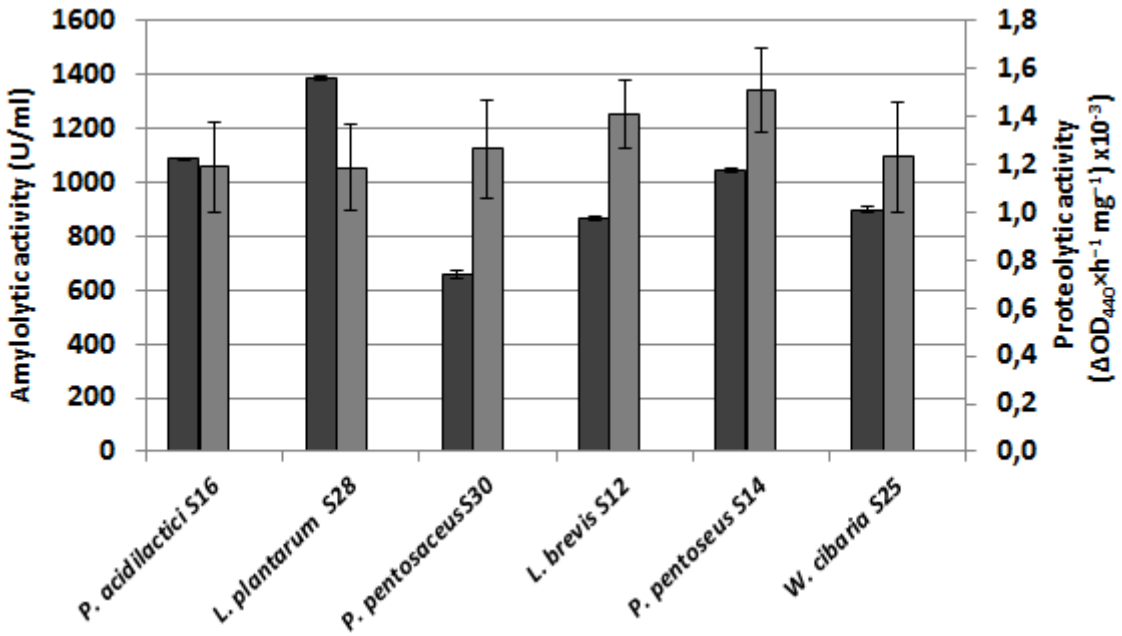

Amylolytic activity (U/ml)
1600
1400
1200
1000
800
600
400
200
0
1,8
1,6
1,4
1,2
1,0
0,8
0,6
0,4
0,2
0,0
Proteolytic activity
(ΔOD440xh-1 mg-1) x10-3)
P. acidilactici S16
L. plantarum S28
P. pentosaceusS30
L. brevis S12
P. pentoseus S14
W. cibaria S25

Fig. 5

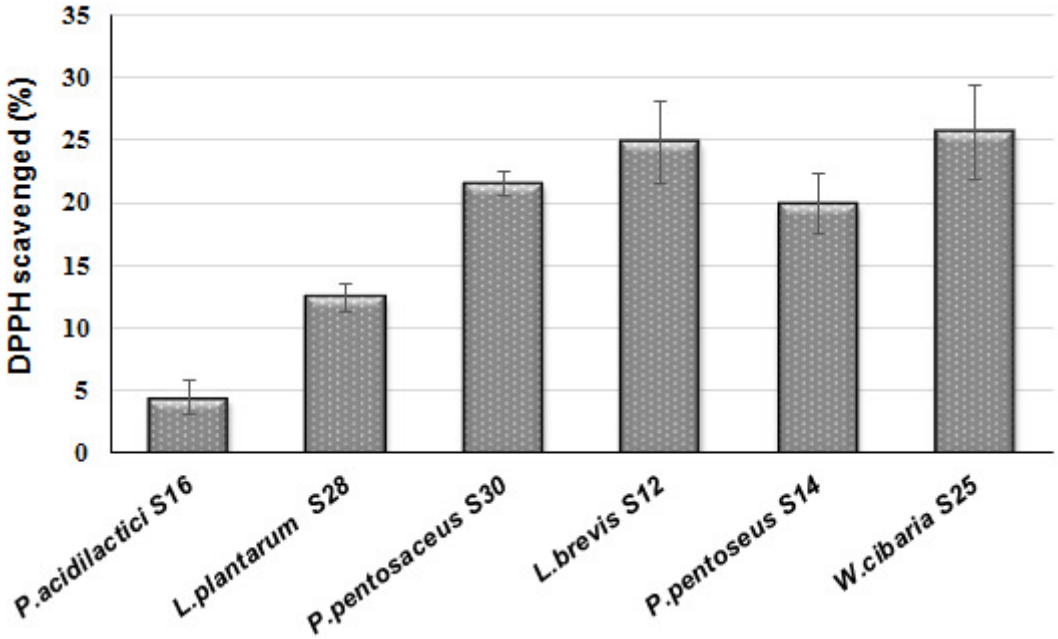

DPPH scavenged (%)
35
30
25
20
15
10
5
0
P.acidilactici S16
L.plantarum S28
P.pentosaceus S30
L.brevis S12
P.pentoseus S14
W.cibaria S25

Fig. 6

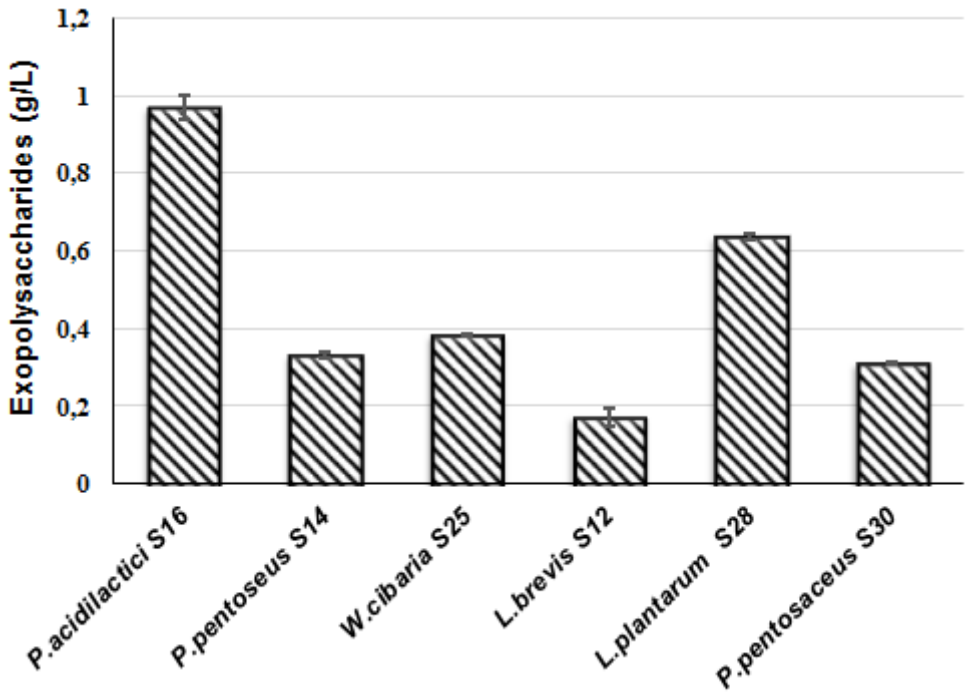
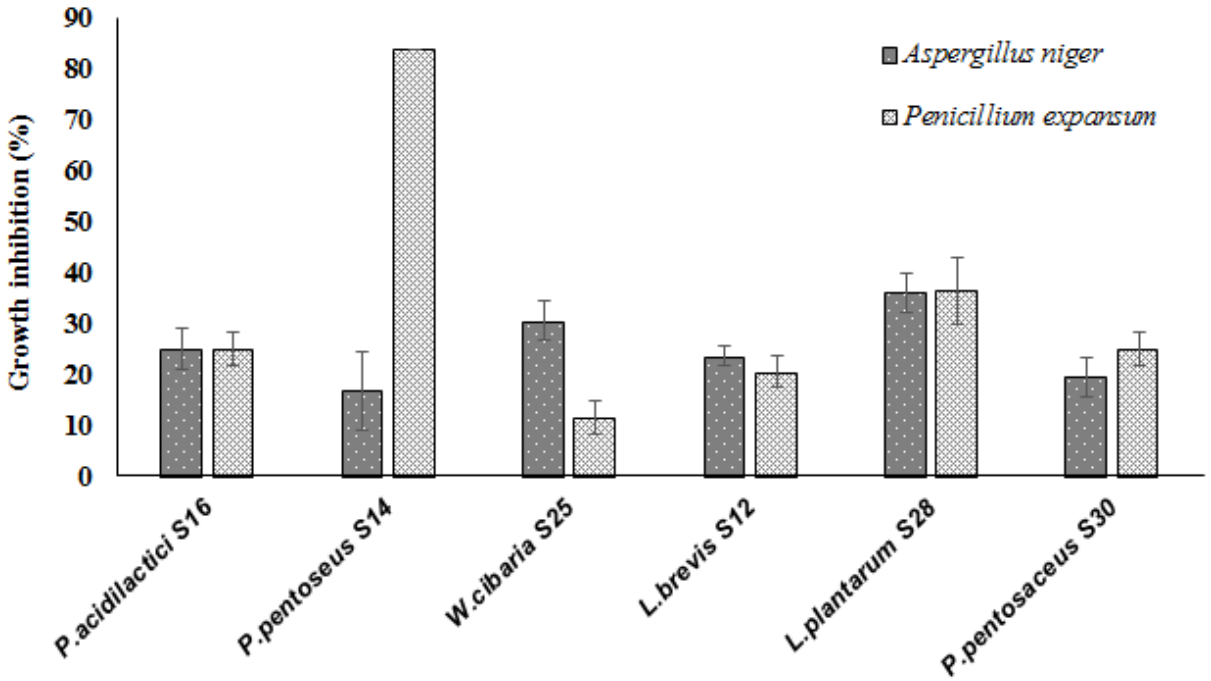

Exopolysaccharides (g/L)
1,2
1
0,8
0,6
0,4
0,2
0
P.acidilactici S16
P.pentoseus S14
W.cibaria S25
L.brevis S12
L.plantarum S28
P.pentosaceus S30

Fig. 7


Growth inhibition (%)
90
80
70
60
50
40
30
20
10
0
Aspergillus niger
Penicillium expansum
P.acidilactici S16
P.pentoseus S14
W.cibaria S25
L.brevis S12
L.plantarum S28
P.pentosaceus S30