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1 **Microbiological analysis and Assessment of biotechnological potential of lactic acid bacteria isolated from**
2 **Tunisian flours**

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58 **Abstract**

59 The present work was carried out to identify lactic acid bacteria (LAB) from wheat flours and to evaluate their
60 technological capabilities for potential incorporation in sourdough process. Six samples of baking wheat flours
61 were collected from mills situated in different geographical regions of Tunisia and used for microbiological
62 analysis of total mesophilic aerobic microorganisms, yeasts, moulds and LAB. A total of 45 autochthonous LAB
63 were isolated and identified by genetic analysis of 16S-23S rRNA intergenic transcribed spacer ITS generated
64 patterns ITS-PCR. One representative strain of each ITS-PCR pattern was subjected to partial sequencing of the
65 16S rRNA. Strains were identified as *Weissella cibaria*, *Lactobacillus plantarum*, *Lactobacillus brevis*,
66 *Pediococcus pentosaceus*, *Pediococcus pentoseus*, *Pediococcus acidilactici*, *Enterococcus faecium*,
67 *Enterococcus casseliflavus* and *Enterococcus faecalis*. Technological features including acidification,
68 antimicrobial, amyolytic, proteolytic and antioxidant activities of six selected LAB strains were investigated for
69 future in situ applications. Moreover, LAB were investigated for their ability to produce exopolysaccharides. All
70 tested LAB showed good acidifying ability by decreasing significantly ($p<0.05$) the pH of flour extract below
71 4.0 after 24 h and below 3.0 after 72 h. *P. pentoseus* and *P. acidilactici* presented fermentation quotient FQ (ratio
72 of lactic and acetic acids) close to the optimal range. All flour LAB isolates, demonstrated extracellular
73 proteolytic activity. *W. cibaria* S25 had the highest radical-scavenging activity with a rate of 25.57%. *L.*
74 *plantarum* S28 demonstrated the highest amyolytic activity (1386 U/mL) followed by *P. acidilactici* S16 (1086
75 U/mL). Although, *L. plantarum* S28 showed the highest production of exopolysaccharides (0.97 g/L). Moreover,
76 different halo of inhibition were detected against *E. coli*, *Staphylococcus aureus*, *Aspergillus niger* and
77 *Penicillium expansum*. This study revealed that autochthonous flour LAB exhibited interesting technological
78 features and thus could be used in sourdough production.

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80 **Keywords:** Baking flour, lactic acid bacteria, acidification, proteolytic, amyolytic, antimicrobial activity.

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96 **Introduction**

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

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

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

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

117 LAB are commonly present in various habitats, including milk and dairy products, vegetables, meat and cereal
118 products, where fermentation can take place (Giraffa 2014) and they constitute the majority of the rate of the
119 commercial starter cultures (Corsetti and Settanni 2007; De Vuyst et al. 2009; Guyot 2010). LAB have been
120 isolated, identified and characterized from cereals (Mamhoud et al. 2016) or mature sourdoughs (Digaitiene et al.
121 2012) or fermented dough (Mhir et al. 2007).

122 However, few studies were focused on the characterization of LAB isolated from wheat flours (Corsetti et al.
123 2007; Alfonzo et al. 2013). To our knowledge, there are no previous studies focused on the characterization of
124 lactic acid bacteria isolated from Tunisian wheat flours.

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

133

134 **Materials and Methods**

135 **Sampling and microbiological analyses**

136 Six samples of baking wheat flours were collected from mills situated in different geographical regions of
137 Tunisia (Nabeul, Tunis, Sousse, Djerba and Sfax), and used for microbiological analysis of total mesophilic
138 aerobic microorganisms, yeasts, moulds and Lactic acid bacteria (LAB) (Table 1). Ten grams of flour were
139 homogenized with 90 mL of sterile peptone water solution (peptone 0.1% and NaCl 0.85%) (Minervini et al.
140 2015). Total mesophilic aerobic microorganisms, yeasts and moulds were enumerated using the PCA (Plate
141 Count Agar) and SD (Sabouraud Dextrose) agar media with chloramphenicol 5%, respectively (Minervini et al.
142 2015). Lactic acid bacteria (LAB) were enumerated using MRS medium (Man, Rogosa, Sharpe agar)
143 supplemented with 0.01% of cycloheximide (MP Biomedicals) to avoid fungal growth and 0.0025% of
144 bromocresol green (MP Biomedicals) to indicate acidification of the medium (yellow turn) by LAB.

145

146 **Isolation of lactic acid bacteria**

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

155

156 **DNA extraction, characterization and molecular identification of lactic acid bacteria**

157 Genomic DNA was extracted according to the method described by Wilson (2001) using CTAB/NaCl method.
158 Bacterial strains were characterized genotypically by profile analysis of the 16S-23S rDNA Internal Transcribed
159 Spacer (ITS) region using universal primers (s-d-bact-1494a-20 and s-d-bact-0035-a-15) (Daffonchio et al.
160 1998). Bacteria strains presenting the same band patterns were clustered in the same ITS-haplotype. One or two
161 representative strains from each cluster have been selected for 16S rDNA gene PCR amplification which was
162 performed using universal primers (s-d-bact-0008-a-S-20 and s-d-bact-1495-a-A20) according to Daffonchio et
163 al. (1998). The two PCR were performed on a thermocycler (BioRad) using this program: 94 °C for 3 min, 35
164 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
165 separated by electrophoresis through 1.5% (w/v) agarose gel containing ethidium bromide (0.5 mg/mL).

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

173

174 **Technological characterization of LAB**

175 **Acidification activity**

176 To evaluate the acidification activity of isolated LAB, a sterile flour extract (SFE) was prepared according to the
177 method described by Alfonzo et al (2013): 200 g of flour was suspended in distilled water (1 L) and sterilized at
178 121 °C for 20 min. After precipitation, the flour was removed. The supernatant was used as liquid broth in
179 subsequent experiments. Overnight LAB cultures on MRS broth were centrifuged (5000 x g for 5 min and
180 washed 3 times with sterile distilled water. LAB cells were inoculated in 50 mL of the solution SFE with 1%
181 (v/v) of bacterial suspension at 10⁹ CFU/mL and incubated at 30 °C. The pH measurements were taken initially,
182 after 24 h, 48 h and 72 h of inoculation.

183 Strains that rapidly and greatly decreased SFE pH, were analyzed for their ability to produce lactic and acetic
184 acids, following 24, 48 and 72h of fermentation. Each resultant sample of acidified SFE (aSFE), was mixed with
185 1/10 mixture of ethyl-butyric acid 2 mg mL (internal standard), filtered through 0.2-µm filters and stored at -80
186 °C until analysis. A system composed of a 7020A gas chromatograph (Agilent Technologies Inc., Palo Alto, CA)
187 connected to a mass spectrometry (MS) 5975N detector (Agilent) was used to quantify the short chain fatty acids
188 SCFA. Data were collected with Enhanced ChemStation G1701DA software (Agilent). 1 µl were directly
189 injected into the gas chromatograph equipped with a ZB-WAX capillary column (30-mm length by 250 µm
190 internal diameter, with a 0.25 µm film thickness; Agilent) using H₂ as the gas carrier, with a constant flow rate of
191 1.5 mL/min. The temperature of the injector was kept at 220 °C, and the injection was performed in split less
192 mode. Chromatographic conditions were as follows: an initial oven temperature of 50 °C, 5 °C/min up to 180 °C,
193 1 min at 180 °C, and 20 °C/min up to 220 °C for cleaning the column. The column was directly connected to the
194 MS detector, and the electron impact energy was set to 70 eV. The data collected were in the range of 25 to 250
195 atomic mass units (3.25 scans/s). The organic acids were identified by comparing their mass spectra with those
196 held in the NSIT MS Data Center and 2HP-Wiley 138 library (Agilent) and by comparing their retention times
197 with those of the corresponding standards (Volatile Free fatty acid mixture CRM46975) purchased from Sigma.
198 The peaks were quantified as the relative total ionic count abundance with respect to the internal standard. The
199 concentration estimated in mM of each acid was calculated using linear regression equations ($R^2 \geq 0.99$) from the
200 corresponding curves of standards obtained with eight different concentrations.

201

202 **Proteolytic activity**

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

207

208 **Antioxidant activity**

209 The antioxidant activity was measured using DPPH according to the method described by Lin and Chang (2000).
210 LAB were harvested by centrifugation at 4400 x g for 10 min after culture of 18 h incubation at 37 °C. The test
211 was performed on the intact bacterial cells. Cells were washed three times with phosphate buffer solution (PBS:
212 0.85% NaCl, 2.68 mM KCl, 10 mM Na₂HPO₄, and 1.76 mM KH₂PO₄, pH 7.4) and resuspended in the same
213 buffer. The sample (0.8 mL) was reacted with 1 mL DPPH solution (0.2 mM in 0.5% ethanol). The control was

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

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

217

218 **Amylolytic activity**

219 The amylolytic activity of LAB was measured using the starch-iodine method, described by Bartkiene et al
220 (2013). To evaluate this activity, the sterile flour extract SFE was enriched with glucose 0.15% in order to
221 enhance the bacterial growth. The absorbance was measured at 670 nm using a spectrophotometer (Thermo
222 Scientific Multiskan GO). One unit of α -amylase activity (1 AU) was defined as an amount of enzyme that
223 catalyzes 1 g soluble starch hydrolysis to dextrins in 10 min at 30 °C temperature.

224

225 **Antimicrobial activity**

226 The antimicrobial activities of LAB were determined against pathogenic bacterial and fungal strains.
227 *Enterococcus faecalis* ATCC 29212, *Escherichia coli* DH5 α , *Pseudomonas aeruginosa* ATCC 27853 and
228 *Staphylococcus aureus* ATCC 25923 were used as indicator strains. These pathogens were grown in Brain heart
229 infusion (BHI) broth at 37 °C. The inhibitory activity was evaluated with spots method. The colonies of LAB
230 were grown in MRS medium incubated for 24 h at 30 °C. The indicator strains were inoculated at 10⁵ CFU/mL
231 in 5 mL of soft agar (containing 0.75% agar) and poured into petri dishes which were then incubated
232 anaerobically at 37 °C for 24 h. Antimicrobial activity was expressed as inhibition diameter zones in millimeter
233 (mm) against the pathological strains. The zone of inhibition was divided as follows: strong inhibitor (d \geq 15
234 mm), medium inhibitor (11 \leq d < 15 mm), no significant inhibitory effect (d < 11 mm).

235 The antifungal activity of LAB was evaluated against two fungi *Aspergillus niger* and *Penicillium expansum*
236 according to the method described by Whipps (1987). LAB were inoculated on the modified MRS agar (without
237 ammonium citrate and sodium acetate), 2 cm from the edge line of the petri dish and then grown at 30 °C for 48
238 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.
239 Control plates were inoculated with fungi only. The antifungal activity of LAB was expressed according to the
240 following formula:

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

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

243

244 **Production of exopolysaccharides**

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

249

250 **Statistical analysis**

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

255

256 **Results and discussion**

257 **Microflora of wheat flour**

258 Examination of the microbiological quality of wheat flour samples was performed in order to gain a common
259 view about the hygienic quality and microbiological load. The microbial content of flours is composed of
260 mesophilic aerobic bacteria, yeasts, moulds, and lactic acid bacteria. The results of microbiological analysis
261 showed that yeasts constituted the major microbiota of the flour samples (Table 1). Yeast counts ranged from
262 0.30 to 2.57 Log CFU/g, whereas presumptive LAB counts varied from 0.17 to 1.68 Log CFU/g. The mean
263 mesophilic aerobic bacteria counts in all flour samples studied were 1.53 to 2.11 Log CFU/g. All samples were
264 below the maximum acceptable limits of the Codex Alimentarius (FAO 1995b). According to Minervini et al.
265 (2015), LAB contaminating flour is strongly affected by the endophytic microbiota of cereals, mainly by the
266 plant organs, the cultivars, and the phenological growth stages. A number of plant-associated microorganisms
267 infect grains, and so flours, touching the whole quality of leavened baked goods (De Vuyst et al. 2009; Gobbetti
268 et al. 2014).

269

270 **Isolation and identification of lactic acid bacteria**

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

275 The isolates were subjected to ITS-PCR amplification analysis and 16S-PCR sequencing. In fact, diverse studies
276 have previously described the efficiency of ITS for inter- and intra-differentiation at the genus/ species level
277 (Gürtler and Stanisich 1996) due to the high variability of these spacers. The ITS-PCR amplification generated
278 different patterns (bands ranging from 50 to 1000 base pairs). Comparing the generated patterns of PCR products
279 obtained from the studied isolates, we distinguished 09 ITS fingerprints. One representative of each ITS-PCR
280 pattern were subjected to partial sequencing of the 16S rRNA. LAB isolates were identified at species level by
281 16S rRNA gene sequencing which is generally regarded as a more reliable solution for the classification and
282 identification of LAB (Ehrmann and Vogel 2005). According to the comparison of 16S rDNA sequences with
283 those available in GenBank, all isolates were related to LAB species with sequence homology $>97\%$.
284 Phylogenetic tree of LAB isolated from wheat flours was constructed based on the 16S rDNA sequences from
285 evolutionary distances by the neighbor-joining method (Figure 1). LAB identification showed several consistent
286 recognized species that are affiliated to four lactic genotypic groups (Figure 1). LAB strains were identified to:
287 *Weissella cibaria* (05 strains), *Lactobacillus plantarum* (08 strains), *Lactobacillus brevis* (04 strains),
288 *Pediococcus pentosaceus* (10 strains), *Pediococcus pentoseus* (04 strains), *Pediococcus acidilactici* (04 strains),

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

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

297 The dominant species of *Lactobacillus* found from wheat flours was *L. plantarum* followed by *L. brevis*. This
298 finding reinforced the concept that *L. plantarum* has a strong ecological or metabolic adaptability to different
299 habitats (Alfonzo et al. 2013; Minervini et al. 2015). However, these two *Lactobacillus* species were reported to
300 dominate raw and fermented vegetables (Rhee et al. 2011). Similar findings were noted for *P. pentosaceus*
301 (Yang and Chang 2010; Swain et al. 2014). Together with *L. plantarum* and *W. cibaria* are generally isolated
302 from plants, wheat grains and fermented wheat products (sourdough) (Corsetti et al. 2007; Trias et al. 2008;
303 Alfonzo et al. 2013). For *Enterococcus* genus, *En. faecium* was the most commonly species presented followed
304 by *En. casseliflavus* and *En. faecalis*. In fact, *enterococci* are normal inhabitants of gastrointestinal tract (Giraffa
305 2002). The isolated *Enterococcus* strains were discarded from further studies to avoid risks for antibiotic
306 resistance and virulence gene dissemination which contribute to the pathogenesis of virulent bacteria (Bortolaia
307 et al. 2016). *Weissella* genus was represented at low occurrence by only *W. cibaria* species. Recording to our
308 findings, there was no correlation between LAB species and the geographical location of the wheat flour
309 samples.

310

311 **Assessment of the technological properties of LAB isolates**

312 **Acidification activity**

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

328 The concentration of lactic and acetic acids in SFE was carried out on four strains (*P. acidilactici*, *P. pentoseus*,
329 *L. plantarum* and *L. brevis*), that displayed interesting technological properties, after 24, 48 and 72 h of
330 fermentation (Figure 3). Lactic acid concentration increased after 48 h for all LAB strains. It reached 5.73 - 8.36
331 mg/g. After 72 h, the content increased significantly ($p<0.05$) and reached 7.77 mg/g for *P. acidilactici* and 7.06
332 mg/g for *P. pentoseus*. However, we noted a significant ($p<0.05$) decline of lactic acid concentration after 72h
333 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
334 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)
335 or the degradation of lactic acid produced (Oude Elferink et al. 2001) which may justify the reduction of lactic
336 acid concentration in SFE extract of *L. plantarum* and *L. brevis*

337 The strains displayed a fermentation quotient (FQ: molar ratio between lactic and acetic acids) varied from 0.84
338 to 2.38, which is considered to affect the organoleptic features (aroma and texture) and to prevent ripeness and
339 fungi spoilage of final products and so to guarantee longer shelf life (Gobbetti et al. 2000; Datta and Henry
340 2006). *P. pentoseus* S14 and *P. acidilactici* S16 presented FQ close to the optimal range of 2.0– 2.7 suggested by
341 Hammes and Gänzle (1998).

342

343 **Proteolytic activity**

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

355

356 **Antioxidant activity**

357 The DPPH scavenging activity of the six LAB strains from flour samples are shown in Figure 5. The results
358 showed DPPH scavenging activity ranging between 4.39 to 25.57%. Among the tested strains, *W. cibaria* S25
359 had significantly ($p<0.05$) the highest radical-scavenging activity with a rate of 25.57%, followed by *L. brevis*
360 S12 with 24.81% and *P. pentosaceus* S30 with 21.49%. Several authors have reported that the fermentation by
361 lactic acid bacteria with antioxidant activity is considered as one of the most important tool suitable to enhance
362 the functional and the organoleptic potential of several fermented cereal flours (Coda et al. 2012; Rizzello et al.
363 2013; Curiel et al. 2015).

364 **Amylolytic activity**

365 The biodiversity of amylolytic lactobacilli, ubiquitously used in cereals processing, is quite limited (Reddy et al
366 2008). In this work, the *in-vitro* amylolytic activity of the tested LAB was evaluated by starch-iodine method.
367 According to the results presented in Figure 4, all tested LAB are characterized by starch hydrolysis. They

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

388

389 **Exopolysaccharide production**

390 The cell-free supernatants of selected LAB strains were examined for the amounts of exopolysaccharide (EPS)
391 yields using phenol-sulfuric acid method. EPS production of each strain was presented in Figure 6. EPS amount
392 produced by selected LAB isolates ranged from 0.172 to 0.970 g/L. Figure 6 obviously demonstrates that the
393 strains *P. acidilactici* S16 and *L. plantarum* S28 showed significantly ($p < 0.05$) highest EPS production (0.97
394 and 0.636 g/L, respectively). Several health benefits have been attributed to exopolysaccharides EPS from LAB
395 which are used to improve the textural properties of fermented foods (Crescenzi 1995; Ruas-Madiedo et al.
396 2008). These strains can be used in sourdough and may have applications in bread making.

397

398 **Antibacterial activity**

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

408 *brevis* S12 and *P. pentosaceus* S30 presented significantly ($p<0.05$) a medium growth inhibition activity against
409 *En.faecalis* with an average of 11 mm. In our case, we observe that the inhibition of microbial growth by the six
410 LAB is due to pH variation on culture media and might be a result of fermentative compounds accumulation.
411 LAB are known to produce a range of metabolic end compounds that are able of interfering with the growth of
412 certain undesirable microbes in food systems (Vandenbergh 1993; Alvarado et al. 2006). In fact, most of the
413 inhibitory activity exhibited by LAB strains was attributed to pH reduction by organic acids. Besides, it is
414 suggested that acid products interfere with permeability of plasmic membrane and raise its diffusion leading to
415 stop metabolic activities and so to the inhibition effect of sensitive microorganisms (Piard and Desmazeaud
416 1991). So, these findings led to suggest that sourdough and bread produced with these tested LAB strains,
417 showing consistent ability to retard the growth of both pathogen moulds and bacteria species, thus have the
418 potential to improve the shelf-life of wheat bread.

419

420 **Antifungal activity**

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

434

435

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441

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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	Couscouseries 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.

	<i>Enterococcus faecalis</i> ATCC 29212	<i>Pseudomonas aeruginosa</i> ATCC 27853	<i>Escherichia coli</i> DH5 α	<i>Staphylococcus aureus</i> ATCC 25923
<i>Pediococcus acidilactici</i> S16	8.5 \pm 0.71 ^a	11.5 \pm 0.71 ^b	8 \pm 0 ^a	10.75 \pm 0.35 ^a
<i>Pediococcus pentoseus</i> S14	10 \pm 0 ^b	16 \pm 0 ^a	12 \pm 1.41 ^b	11.75 \pm 1.06 ^b
<i>Weissella cibaria</i> S25	8 \pm 0 ^a	17 \pm 0 ^a	11 \pm 0.71 ^b	12 \pm 0 ^b
<i>Lactobacillus brevis</i> S12	11.5 \pm 0.71 ^b	15 \pm 0 ^a	11 \pm 0 ^b	14.5 \pm 0.71 ^c
<i>Lactobacillus plantarum</i> S28	9 \pm 1.41 ^a	13 \pm 0 ^a	11 \pm 1.41 ^b	12.5 \pm 0 ^b
<i>Pediococcus pentosaceus</i> S30	11 \pm 0 ^b	18 \pm 0 ^a	11.5 \pm 0.71 ^b	11.75 \pm 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.

672 **Figure captions**

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

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

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

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

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

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

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

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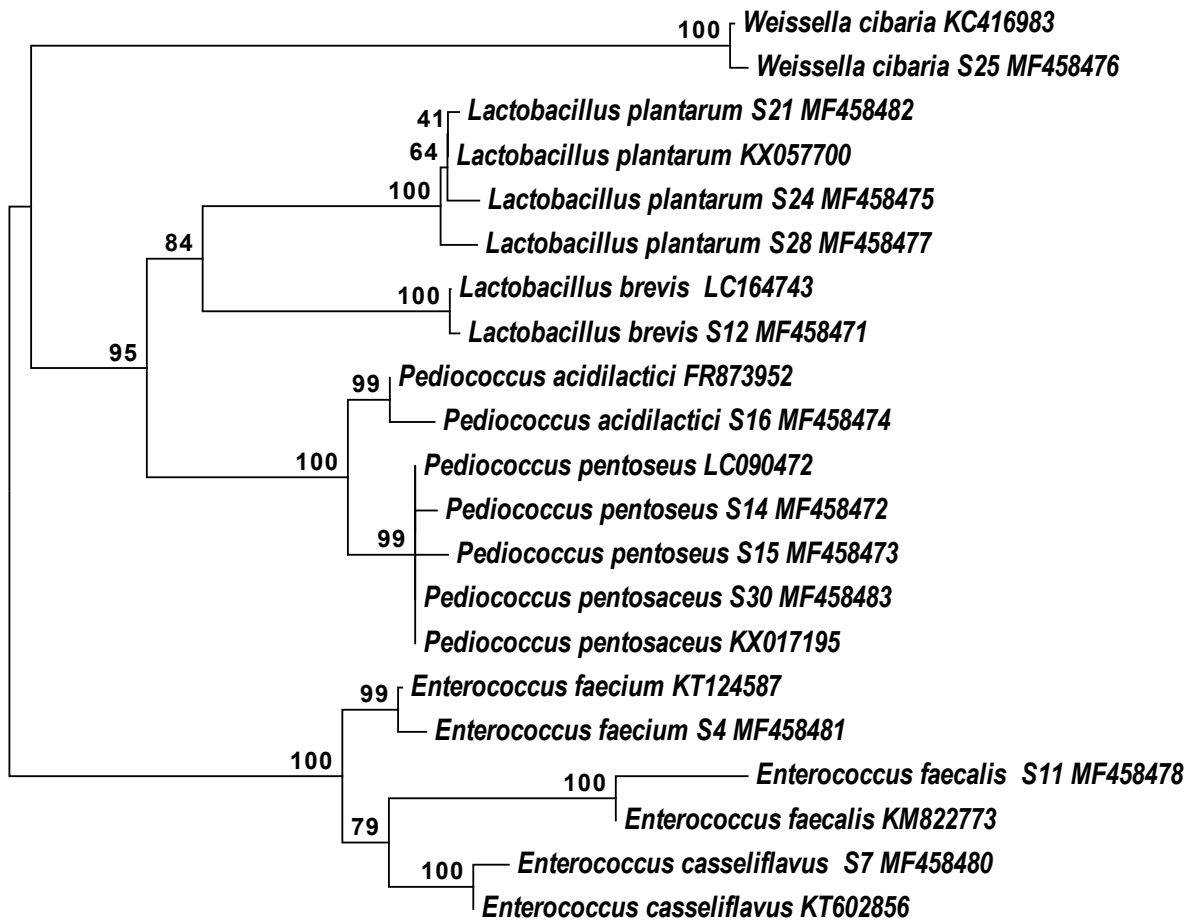
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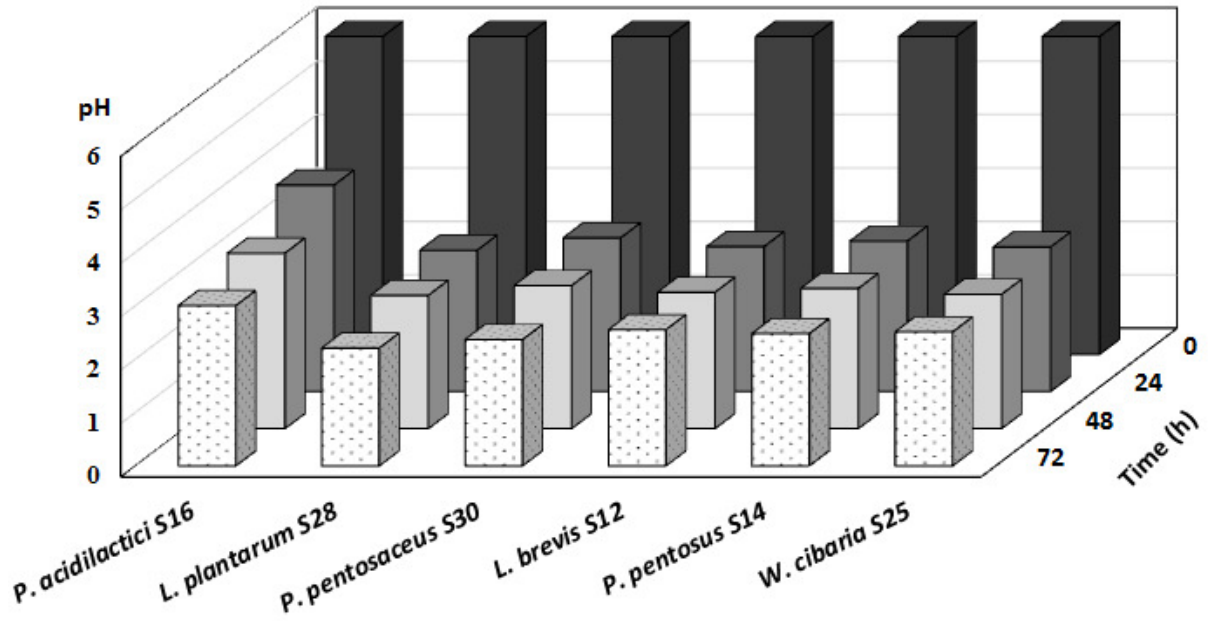
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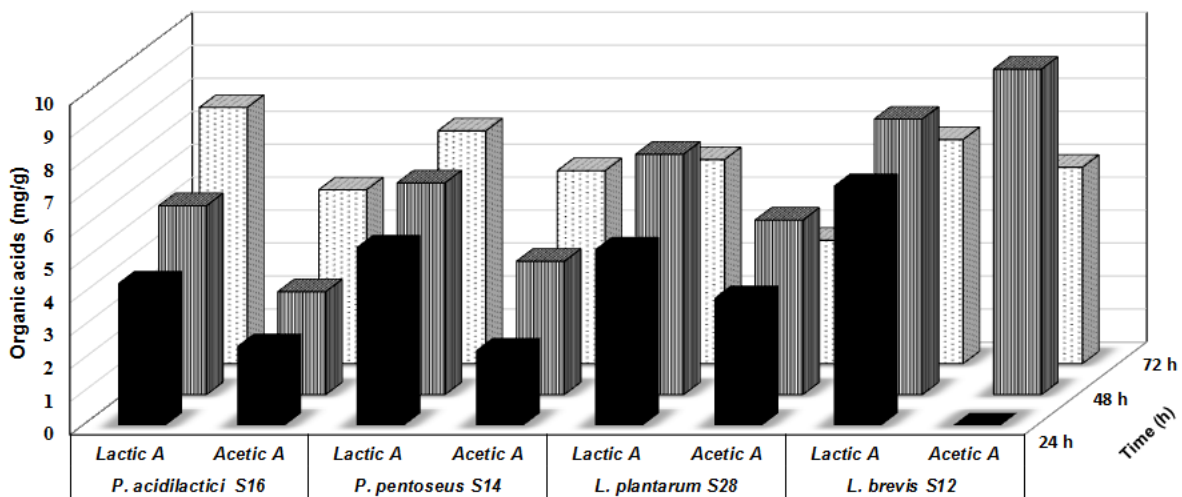
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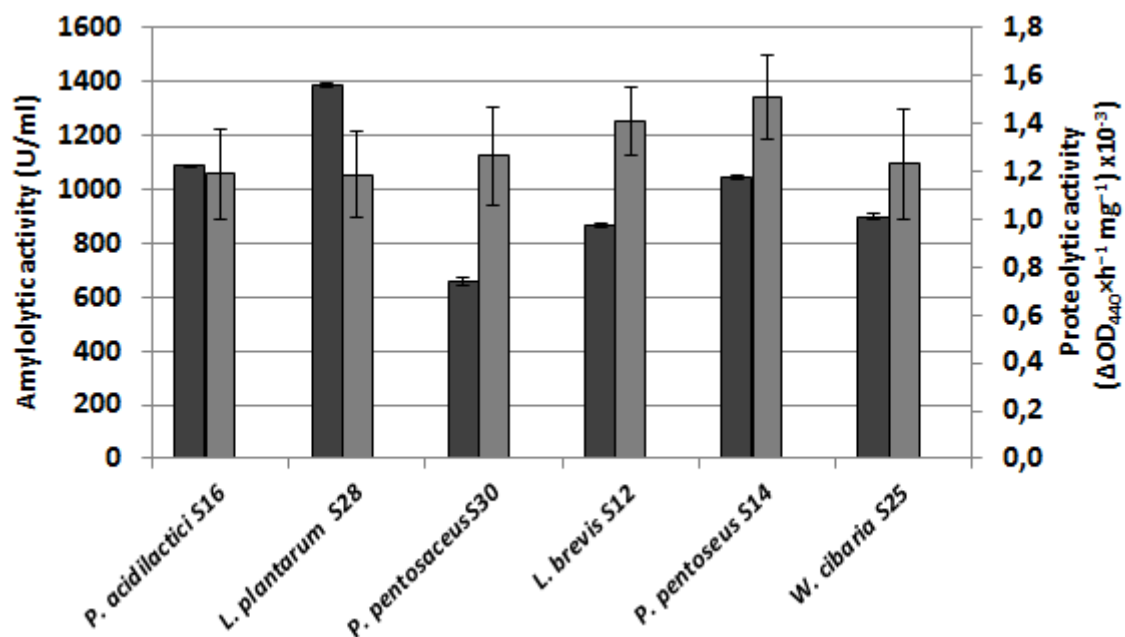
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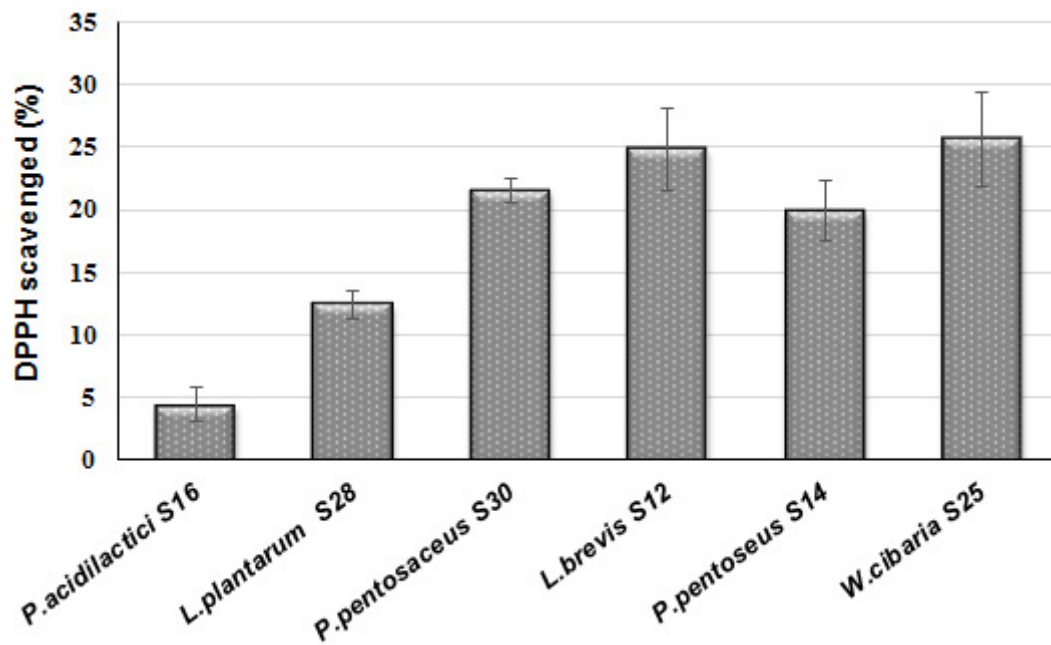
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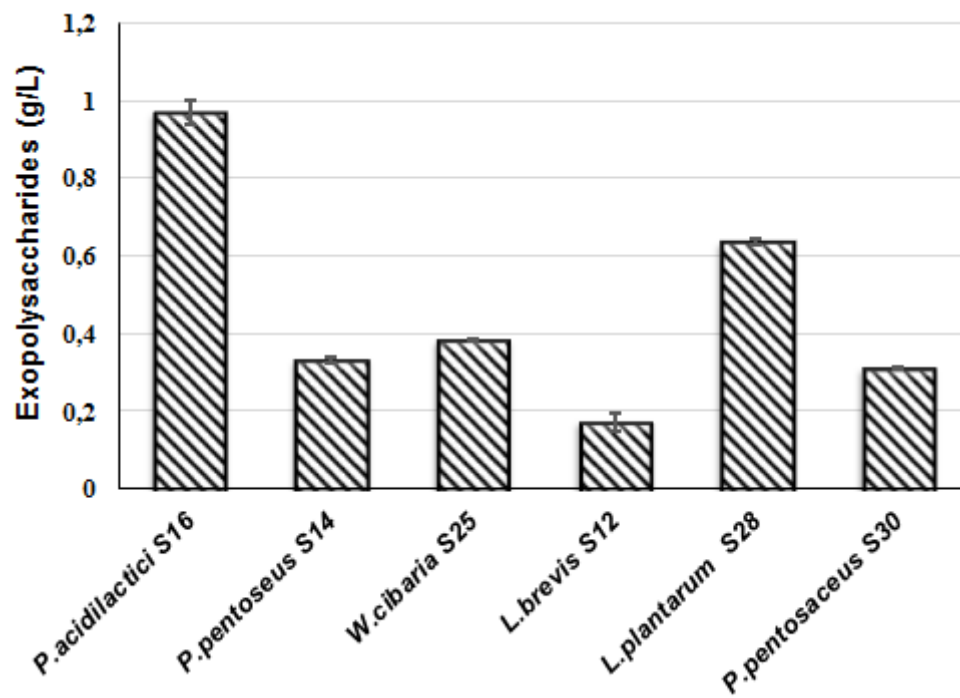
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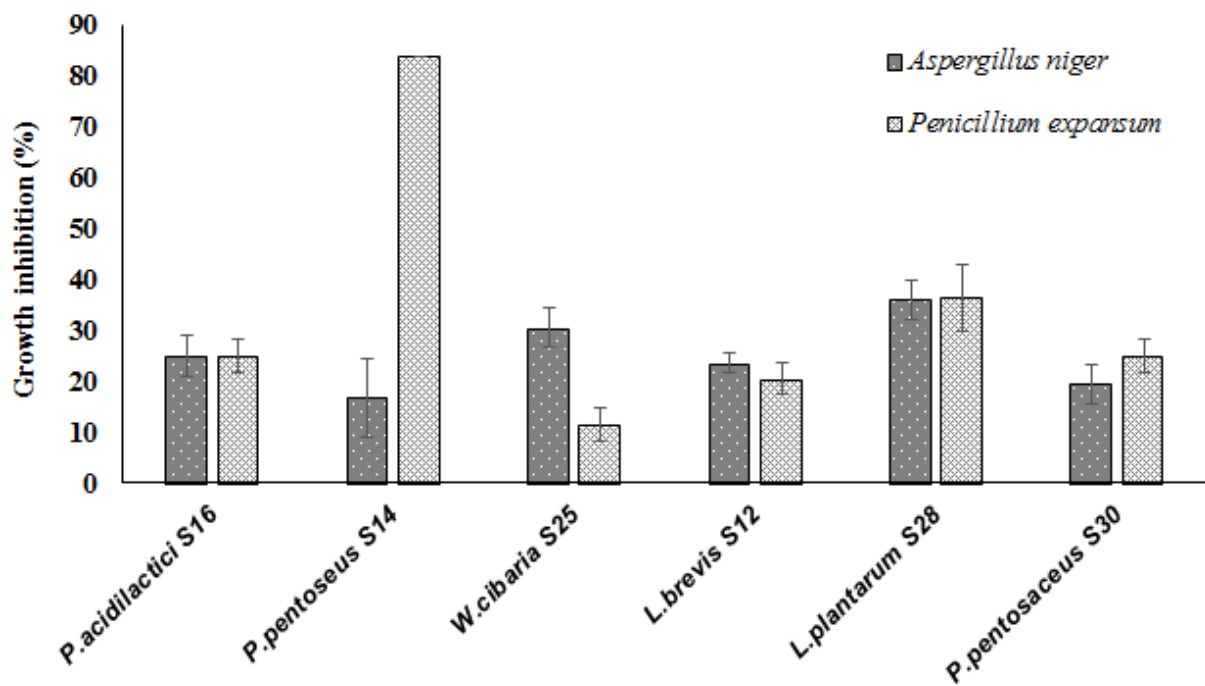
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868 Fig. 7

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