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ORIGINAL ARTICLE

Microbial community dynamic in tomato fruit during spontaneous fermentation and biotechnological characterization of indigenous lactic acid bacteria

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Abstract

The present work aimed to study the microbial dynamics in tomato fruits under spontaneous fermentation process and the biotechnological properties of lactic acid bacteria (LAB) for future starter culture formulation. The isolation of native microbial species was performed using diverse specific media. Microbial identification was performed using the variability analysis and sequencing of ribosomal DNA-amplified fragments. Moreover, LAB ($n = 85$) were evaluated for several physiological and technological characteristics, including salinity and temperature tolerance, esculin and arginine hydrolysis, carbohydrate fermentation, exopolysaccharide production, and antagonistic activity. As well, lycopene, flavonoid, and antioxidant compounds were determined in fermented tomato samples. Bacterial isolates were assigned to ten bacterial genera, namely, *Microbacterium*, *Bacillus*, *Staphylococcus*, *Pantoea*, *Flavobacterium*, *Enterobacter*, and *Citrobacter* including three genera of LAB: *Lactobacillus*, *Leuconostoc*, and *Enterococcus.* Moreover, the amplification of ITS1-5.8S-ITS2 regions allowed the detection of *Aspergillus fumigatus* and three species of yeast: *Candida carpophila*, *Meyerozyma caribbica*, and *Wickerhamomyces onychis*. During the spontaneous fermentation, the majority of spoilage bacteria and fungi completely disappeared after the third week, whereas LAB and yeast remain until the end of fermentation. LAB exhibited important technological features and high antibacterial activity against human and food-borne pathogenic bacteria. Furthermore, LAB produced various antioxidants for fermented food products. Promising results of this study allowed the identification of the major encountered taxa during spontaneous fermentation of tomato and underline the importance of LAB as a starter culture to achieve microbiologically safe products providing prolonged stability and flavor of vegetable-derived foods.

Keywords Microbial dynamics · Spontaneous fermentation · Lactic acid bacteria · Biotechnological properties

Introduction

Tomato vegetables (*Solanum lycopersicum*) are among the most cultivated and eaten foods in the world. It presents a source of water, antioxidants (lycopene and β-carotene), vitamin C, polyphenols, flavonoid, phytosterols, dietary fibers, and mineral elements for human diet (Gebbers 2007). Antioxidants play an essential role in fighting free radical attacks leading to cell damage and human body problems. Moreover, antioxidants prevent several chronic diseases, including cancer, neurodegenerative, cardiovascular, asthma, and also in improving the function of immune system (Montet et al. 2014).

Tomatoes, and other fresh vegetables, have a short shelf-life due to the presence of a high water activity and spoilage microorganisms. Fermentation process was adopted to enhance the vegetable property shelf-life and maintain the safety of foods (Piasecka-Józwiak et al. 2013).

Lactic acid bacteria (LAB) were the main encountered bacteria during spontaneous fermentation (Paramithiotis et al. 2014). This fact may be due to their antimicrobial properties through lactic acid, hydrogen peroxide, diacetyl, and bacteriocin release. Hence, LAB application was reported as a suitable and easy biotechnology to prevent fruit alteration (Di Cagno et al. 2013). Moreover, LAB have attracted food

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industries for their guaranteed safety and were mostly used as bio-preservatives in form of starter cultures. Nevertheless, the application of LAB in fresh fruits and vegetables has not been well developed (Lee et al. 2011; Wouters et al. 2013).

The present study aimed to assess the microbial dynamic during spontaneous fermentation process and to investigate the biotechnological characteristics of LAB, in view, as future starter culture development.

Material and methods

Sampling

Spontaneous fermentation was performed on tomato fruits (*Solanum lycopersicum* L.). Samples were collected in March 2015 from greenhouse of Borj el Amri (Tunisia). Tomatoes were taken aseptically and packaged in plastic seal bags, then brought, at ambient temperature, to the laboratory for experiments.

Fermentation processing and microbiological analysis

Five kilograms of tomatoes was suspended in 5 L of solution containing NaCl (35 g/L) and sucrose (20 g/L) for fermentation process, during 1 month at room temperature (Wouters et al. 2012). To assess microbial dynamics during spontaneous fermentation, each week, 1 g of tomatoes was placed in saline peptone solution (NaCl (0.85%) and peptone (1%), then, mechanically homogenized in a stomacher (Alegre et al. 2011). The obtained suspensions were diluted in tenfold series and plated onto different culture media: tryptic soy agar (TSA) (30 °C for 48–72 h), Man-Rogosa-Sharpe (MRS) agar medium for LAB growth (30 °C for 48–72 h), plate count agar (PCA) medium for mesophilic aerobic microbes (30 °C for 48–72 h). Fungal and yeast were assessed on potato dextrose agar (PDA) medium (25 °C for 5–6 days). Violet red bile lactose (VRBL) agar medium was also used for coliform enumeration (30 °C for 48–72 h) (Alegre et al. 2011; Wouters et al. 2012). After incubation, microorganisms presenting different phenotypes were enumerated, selected, and purified on the same medium. Pure bacterial cultures were stored at − 80 °C in the different broth media containing 25% glycerol. Fungi and yeast were conserved in PDB and yeast glucose peptone (YGP), respectively.

Identification of microbial community

Genomic DNA was extracted from bacterial isolates using two protocols: boiling lysis (Ferjani et al. 2015) and phenol-chloroform method for LAB as detailed by Abed (2013).

Fungal DNA was extracted using the method described by Atoui et al. (2012). A solvent extraction method using phenol/chloroform mixture was used for yeast DNA isolation (Silva-Filho 2003).

The bacterial collection was dereplicated by fingerprinting analysis of the rRNA 16S-23S intergenic transcribed spacer (ITS) region. ITS-PCR amplification was carried out using universal primers S-DBact-0008-a-S-20 and S-D-Bact-1495-a-S-20 (Daffonchio et al. 1998). By visual comparison of the ITS profiles, isolates have been divided onto groups exhibiting the same profile and which correspond to different species of the community. One or more strains for each ITS group has been selected for subsequent identification using16S rRNA gene sequencing**.** Partial 16S rRNA gene was amplified from selected strains. Fungal and yeast identification was performed on the basis of the ITS sequence using ITS1 (50-TCCGTAGGTGAACCTGCGG-30) and ITS4 (50-TCCTCCGCTTATTGATATGC-30) primers targeting ITS1-5.8S-ITS2 regions for amplification (Lin et al. 2005). Amplicons were purified with Exonuclease-I and Shrimp Alkaline Phosphatase (Exo-Sap, Fermentas, Life Sciences) following the manufacturer's standard protocol. Sequence analysis of purified DNA was performed using a Big Dye Terminator cycle sequencing kit V3.1 (Applied Biosystems) and an Applied Biosystems 3130XL Capillary DNA Sequencer. Sequences were analyzed by BLAST and compared with those available at the National Centre for Biotechnology Information (NCBI) database (http://www.ncbi.nlm.nih.gov) and Ribosomal Database Project (RDP) (https://rdp.cme.msu.edu/). The sequences were submitted to the NCBI nucleotide database under the accession number MH037132 to MH037147 and MH107771 for bacterial strains and MH050335 to MH050338 for fungi.

Physiological and technological properties of lactic acid bacteria

LAB were tested for their abilities to support different concentrations of NaCl (4%, 6%, 8%, and 10%), varied temperature (4 °C, 15 °C, 30 °C, 37 °C, and 45 °C), and pH (4, 6, 8, and 10) on MRS agar medium (Khedid et al. 2009). Esculin and arginine hydrolysis were checked on bile esculin agar and M16BCP media, respectively (Thomas 1973; Weiss et al. 2005).

The analysis of carbohydrate fermentation was determined following the protocol described by Samelis et al. (1994), using MRS broth added with different carbohydrate sources: fructose, inositol, galactose, glucose, lactose, maltose, mannitol, mannose, sucrose, sorbitol, and xylose. Proteolytic activity was assessed by streaking LAB on MRS agar medium containing 4% of skimmed milk. A clear halo around the colonies indicates the ability of the strains to produce proteases (Fhoula et al. 2013). Production of aromatic compounds acetoin and diacetyl by LAB was tested on Clark and Lubs medium using Voges–Proskauer assay, as described by De Almeida Júnior et al. (2015).

Tyrosine decarboxylase activity of LAB was revealed on tyramine production medium (TPM) at a pH = 5.5 as detailed by Landete et al. (2007).

The production of exopolysaccharides (EPS) of LAB was tested qualitatively on MRS agar-modified medium as reported by Madiedo and de los Reyes-Gavilan (2005). Besides, EPS were quantified using the phenol-sulfuric method of Dubois et al. (1956).

Screening of antagonistic activity of LAB

The antibacterial activity test was performed in vitro using the agar-well-diffusion method reported by Tagg and McGivern (1971) against *Citrobacter farmeri*, *Staphylococcus haemolyticus*, *Enterobacter cloacae*, *Acinetobacter johnsonii*, *Escherichia coli*, *Bacillus cereus*, *Salmonella enteritidis*, *Staphylococcus aureus MRSA*, *Listeria monocytogenes*, *Enterococcus faecalis*, and *Enterococcus faecium*. Indicative strains were obtained from Laboratory of Microorganisms and Active Biomolecules (LMBA) Tunisia and School of Biotechnology, Catholic University of Portugal. Antibacterial activity was recorded by the distinction of translucent halo zone around LAB and measuring of inhibition zone diameters. According to the diameter of inhibition (mm), LAB were grouped as strains with weak inhibitory activity ($d \leq 12$), medium activity ($12 \leq d \leq 15$), and strong inhibitory activity ($d > 15$). All antibacterial tests were performed in triplicate.

Lycopene, flavonoid, and antioxidant compound determination

Lycopene was quantified on triplicate by calorimetric method according to Periago et al. (2004). Each week, during spontaneous fermentation, 1 g of tomato sample was recovered and homogenized in a stomacher blender, then, suspended in hexane-ethanol acetone (2:1:1) solution. After shaking, a polar and nonpolar layer containing lycopene was distinguished. The total lycopene content was quantified by reading the absorbance of the lycopene hexane solution at 472 nm. A calibration curve was performed using pure lycopene. The total of flavonoid content was estimated following the protocol detailed by Zhishen et al. (1999). Moreover, the total phenolic contents of tomato samples were determined by the Folin-Ciocalteu method (Meda et al. 2005). Briefly, 0.1 ml of Folin-Ciocalteu reagent was added to each tomato extract followed by Na_2CO_3 (20%) solution, 3 min later. The absorbance of the mixture was recorded at 760 nm. Gallic acid was used as standard to generate the calibration curve.

Antioxidant activity of tomato fruits was performed on triplicate according to the thiocyanate method (Mitsuda et al. 1966). Briefly, each sample was suspended on distilled water and mixed with 2.5 ml of linoleic acid and 2 ml of phosphate-buffered saline, then, incubated in darkness at 37 °C. The amount of peroxide was determined by measuring the absorbance at 500 nm after coloring with $FeCl_2$ and thiocyanate.

Results and discussion

Bacterial and fungal isolation and identification

A total of 87 bacterial isolates were selected from TSA, PCA, and VRBL media and 85 isolates of LAB from MRS medium, during 1 month of spontaneous fermentation of tomato fruits. Phylogenetic redundancy of the collection was reduced by applying ITS-PCR fingerprinting corresponding to the different species/subspecies (Daffonchio et al. 1998). We noted five polymorphic

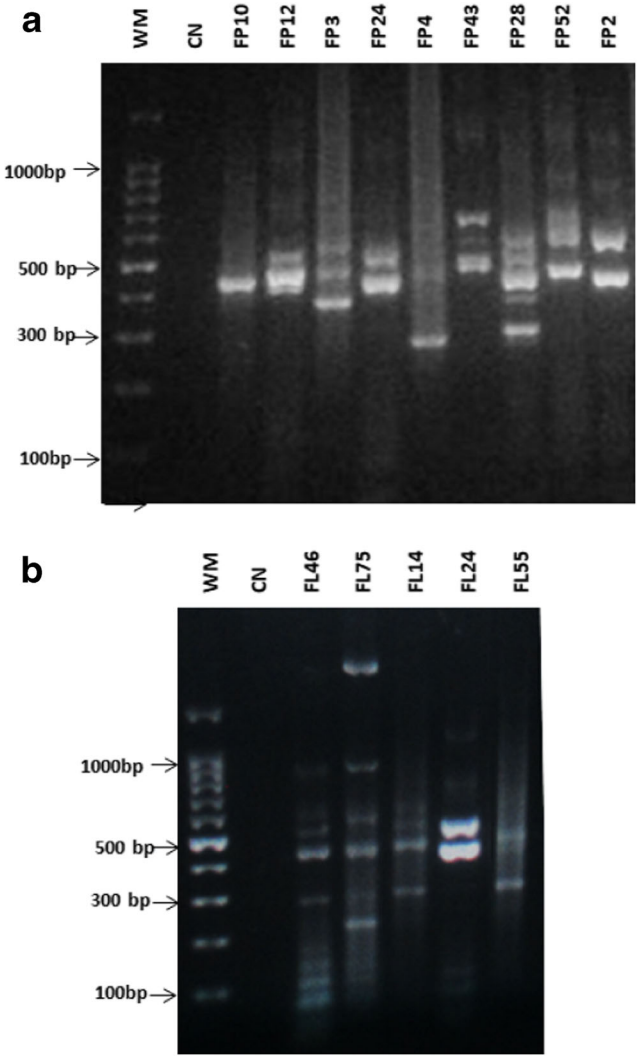

Fig. 1 The ITS-haplotypes of lactic acid bacteria (**a**) and saprophytic/pathogenic bacteria (**b**) of tomato fruits. Mw, molecular weight (100 bp); CN, negative control

haplotypes for LAB and nine for the other bacterial isolates (Fig. 1). Representatives strains of each ITS-PCR haplotype were identified at species level based on 16S rDNA gene sequence analysis. Blast analysis allowed the assignment of the isolates to seven bacterial genera, namely, *Microbacterium*, Bacillus, *Staphylococcus*, *Pantoea*, *Flavobacterium*, *Enterobacter*, and *Citrobacter* and to three genera of LAB: *Lactobacillus*, *Leuconostoc*, and *Enterococcus* (Fig. 2). In line with our findings, the study of Wakil and Dadou (2011), which investigated the microbial diversity of Maize during spontaneous fermentation, confirmed the common occurrence of *Bacillus*, *Staphylococcus*, *Enterobacter*, *Citrobacter*, *Lactobacillus*, and *Enterococcus* genera in fermented vegetables. As well, *Lb. plantarum* and *Ln. mesenteroides* were commonly encountered in spontaneous fermentation of many vegetable-derived products (Maifreni et al. 2004; Paramithiotis et al. 2010; Piasecka-Józwiak et al. 2013. Moreover, *Enterococcus* species have been reported to participate in the fermentation process (Paramithiotis et al. 2010). We recorded an uneven distribution and occurrence of the different bacterial species during the spontaneous fermentation process, as detailed in Fig. 3. During the first week, the bacterial community was dominated by *Ln. mesenteroides* (55.56%) for LAB, accompanied by *Enterobacter ludwigii* (22.5%) and *Staphylococcus haemolyticus* (20%). The Bacillus species occurred during the second week of spontaneous fermentation, while *Staphylococcus haemolyticus* was the most encountered species (34.78%). Several bacterial isolates affiliated to *Pantoea*, *Citrobacter*, *Bacillus*, and *Stahylococcus* species completely disappeared at the third week of the fermentation process, whereas all the LAB species were recorded. At the end of the experiment, the remaining spoilage bacteria were only five species, namely, *Microbacterium arabinogalactanolyticum*, *Staphylococcus saprophyticus*, *Citrobacter farmeri*, *Staphylococcus haemolyticus*, and *Enterobacter* sp., while all LAB species still occurring and *Lb. plantarum* (34.78%) was the major abundant species. Fungi and yeast isolation was also investigated allowing for the detection of *Aspergillus fumigatus* during the first and the second week of spontaneous fermentation and three species of yeast: *Candida carpophila*, *Meyerozyma caribbica*, and *Wickerhamomyces onychis*. Contrary to the other species, *Candida carpophila* was only present at the two first weeks of the fermentation process (Fig. 3).

The obtained results are in agreement with the study of Paramithiotis et al. (2010) which reported the predominance of *Ln. mesenteroides* on tomato fruits at the first step of spontaneous fermentation replaced by *Lb. plantarum* at

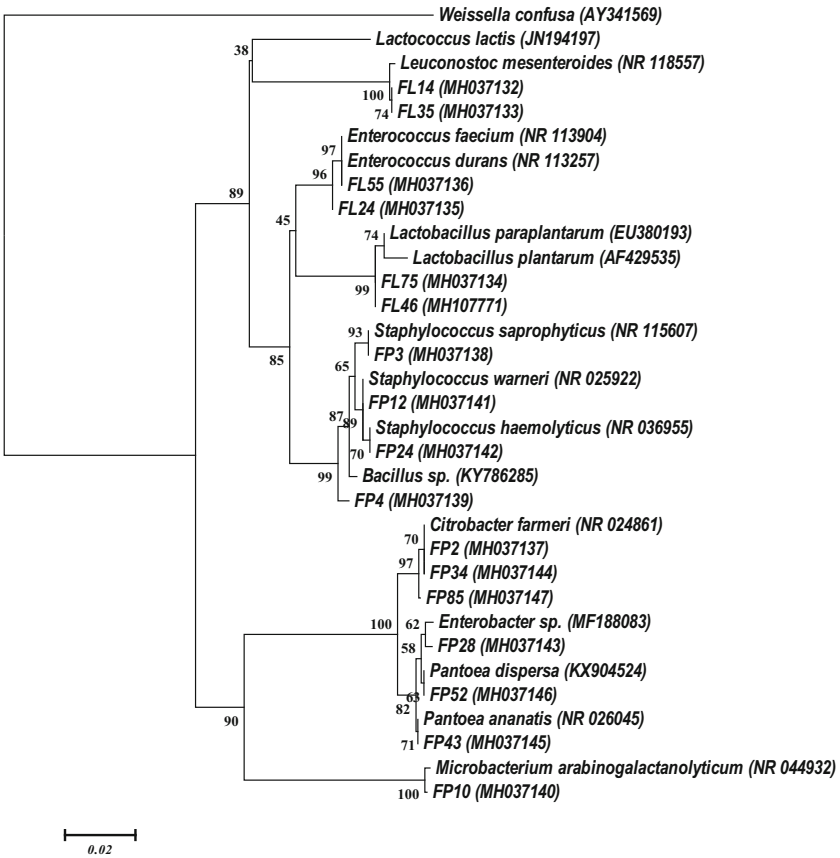

Fig. 2 Phylogenetic tree based on neighbor-joining distance analysis of 16S-rRNA gene sequences. The 16S-rRNA gene sequences were calculated from bootstrap values. GenBank accession numbers are given in parenthesis

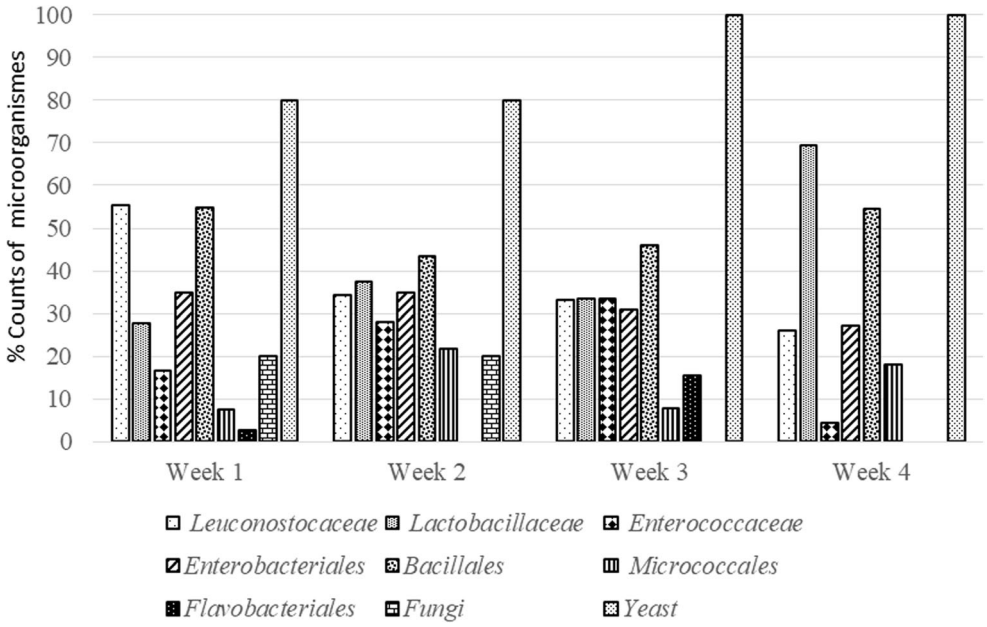

Fig. 3 Diversity of the microbial community on tomato fruits subjected to spontaneous fermentation during 4 weeks

the final stage. Sajur et al. (2007) have previously reported *Leuconostoc* as the predominant LAB on tomato fruit surfaces. The occurrence of LAB species during different phases of spontaneous fermentation suggested the antibacterial and antifungal behavior of LAB. In fact, Harris (1998) reported that lactic fermentation influences the cell numbers of bacterial community during vegetable fermentation.

Physiological and technological properties of lactic acid bacteria

The collection of LAB ($n = 85$) was evaluated for different physiological characteristics. All tested isolates were able to grow in acidic media (pH = 4). A significant fraction of LAB isolates (85.88%) supported a basic media (pH = 8), whereas only 34.11% of LAB were able to tolerate pH = 10 condition.

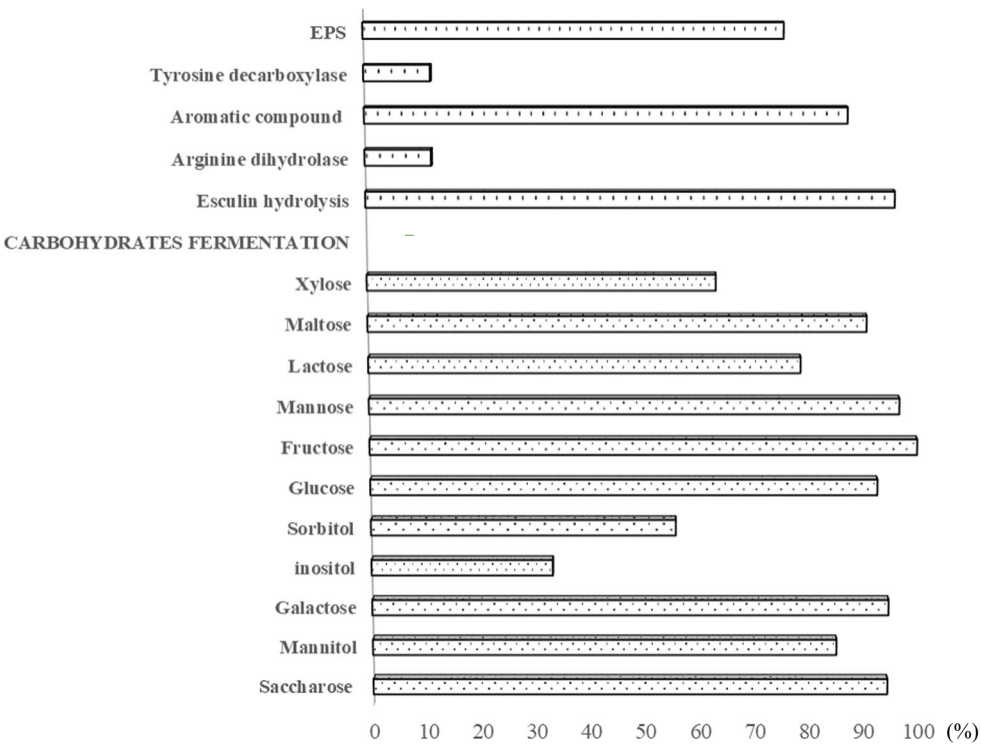

Fig. 4 Percentage of isolates displaying the assayed technological properties in the bacterial collection of lactic acid

Interestingly, 96.47% of investigated LAB resisted to a wide range of temperature (4 °C, 15 °C, 30 °C, 37 °C, and 45 °C). In addition, moderate halo tolerance (8% NaCl) was recorded in 87.05% of the isolates, while only 31.76% tolerated 10% of NaCl.

The growth rate of selected LAB for starter formulation is very important, as it influences their survival and competitive behavior under fermentation process (Piasecka-Józwiak et al. 2013). Further analyses were undertaken to evaluate the technological properties of LAB. The most of LAB isolates (94.11%) exhibited esculin hydrolysis and only few isolates (11.76%), mainly affiliated to *Lactobacillus*, were arginine dihydrolase positive (Fig. 4). The mentioned characteristics above were in accordance with Swan (1954) and Tanasupawat et al. (1998). The analysis of carbohydrate fermentation revealed that more than 90% of LAB were able to ferment eight carbohydrates among the 11 sources tested (sucrose, mannitol, galactose, glucose, fructose, mannose, lactose, and maltose) as detailed in Fig. 4. All evaluated isolates showed a clear zone on MRS medium supplemented with (4%) skim milk, highlighting the importance of proteolytic activity for LAB growth. The evaluation of aroma formation revealed that 85.55% of LAB exhibited aromatic compound production. Moreover, 11.76% of LAB presented tyrosine decarboxylase activity.

Diverse LAB presented desirable technological properties (Table 1). In accordance with Rhee et al. (2011), LAB were able to ferment the sugars to acid and thus lowering the pH, and producing aromatic compounds, providing prolonged stability of food against pathogen proliferation. A significant fraction of the tested isolates (74.11%) were able to form

Table 1 Technological properties of Lactic acid bacteria

Strains codes or accession number (MH)	Closed described species and identity (98–100%)	EPS (mg/ml)	Aromatic compound production	Proteolytic activity	Tyrosine	Arginine	Esculin
1FL5VL	*Ln. mesenteroides*	0	+	+	+	−	+
MH037132	*Ln. mesenteroides*	460.04	+	+	−	−	+
FL19B	*Ln. mesenteroides*	828.81	+	+	−	−	+
FL20	*Ln. mesenteroides*	0	+	+	−	−	+
FL22	*Ln. mesenteroides*	89.84	+	+	−	−	+
FL27	*Ln. mesenteroides*	193.84	+	+	−	−	+
FL30	*Ln. mesenteroides*	46	+	+	−	−	+
FL34	*Ln. mesenteroides*	24.68	+	+	−	+	+
MH037133	*Ln. mesenteroides*	192.63	+	+	−	−	+
FL53	*Ln. mesenteroides*	193	+	+	−	−	+
FL57	*Ln. mesenteroides*	183.32	−	+	−	−	+
FL58	*Ln. mesenteroides*	0.4	−	+	−	+	+
FL71	*Ln. mesenteroides*	192.63	−	+	+	−	−
FL72	*Ln. mesenteroides*	320.92	+	+	−	−	+
FL28	*Lb. plantarum*	241.19	+	+	−	−	+
FL4	*Lb. plantarum*	208.41	−	+	−	+	+
FL12	*Lb. plantarum*	162.7	+	+	−	−	+
FL30CV	*Lb. plantarum*	46.04	+	+	−	−	+
FL36	*Lb. plantarum*	0	+	+	−	−	+
FL46	*Lb. plantarum*	154	+	+	−	+	+
FL60	*Lb. plantarum*	431.08	+	+	−	−	+
FL62	*Lb. plantarum*	162.7	+	+	−	−	+
FL70	*Lb. plantarum*	86.6	+	+	−	−	+
FL77	*Lb. plantarum*	186.56	+	+	−	−	+
MH037134	*Lb. plantarum*	0	+	+	−	−	+
FL78	*Lb. plantarum*	162.7	+	+	−	−	−
MH107771	*Lb. paraplantarum*	154	+	+	−	+	+
MH037136	*En. durans*	554.83	+	+	−	−	+
MH037142	*En. faecium*	816.67	+	+	−	−	+

"+" Positive activity, "−" negative activity

Ln. mesenteroides: *Leuconostoc mesenteroides*, *Lb. plantarum*: *Lactobacillus plantarum*, *Lb. paraplantarum*: *Lactobacillus paraplantarum*, *En. durans*: *Enterococcus durans*, *En. faecium*: *Enterococcus faecium*

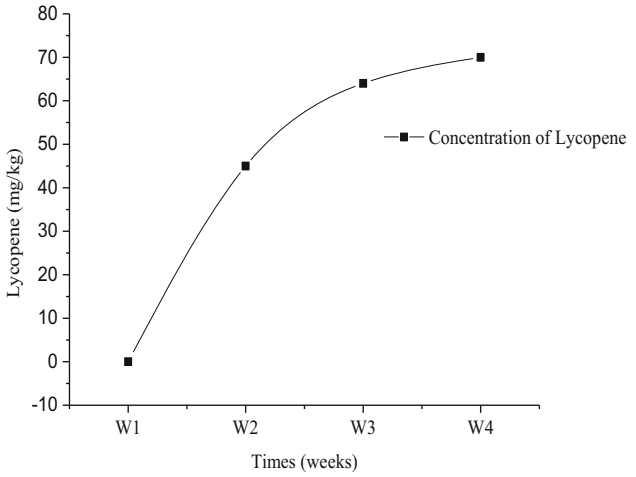

Fig. 5 Lycopene content in tomato fruits during spontaneous fermentation (W1–W4: 4 weeks)

EPS. Positive LAB were further tested quantitatively. Among them, 38.82% produced more than 100 mg/ml of EPS, while EPS production exceeded 300 mg/ml for 10.59% of the bacterial collection. Three LAB strains, namely *Ln. mesenteroides* FL19B, *En. faecium* FL24, and *Lb. plantarum* FL37, were able to release more than 800 mg/ml of EPS. EPS formation was reported as one of the most important researched feature which contribute to the consistency and rheology of fermented products (Cerning et al. 1992; Madiedo and de los Reyes-Gavilan 2005).

Screening of antagonistic activity of LAB

LAB isolates were screened for antibacterial activity against human and food-borne pathogenic bacteria. According to their inhibitory effects on pathogens, LAB were grouped into three classes: weak inhibitor activity with $d \leq 12$ mm, medium activity with diameter 12 mm $\leq d \leq 15$ mm, and strong inhibitor activity, diameter $d > 15$ mm. The results showed that 91.76% of LAB collection presented inhibitory activity against, at least, 7 pathogenic bacteria out of the 11 investigated. Several LAB species exhibited strong inhibitory activity towards *Staphylococcus haemolyticus*, *Enterobacter cloacae*, *Acinetobacter johnsonii*, and *Bacillus cereus*. Among those LAB species, FL8 and FL15S affiliated to *Ln. mesenteroides* and *Enterococcus* genus, respectively, presented strong inhibitory activity against four of the tested pathogenic bacteria. According to Aguilar et al. (2011), the antibacterial activity against pathogenic bacteria

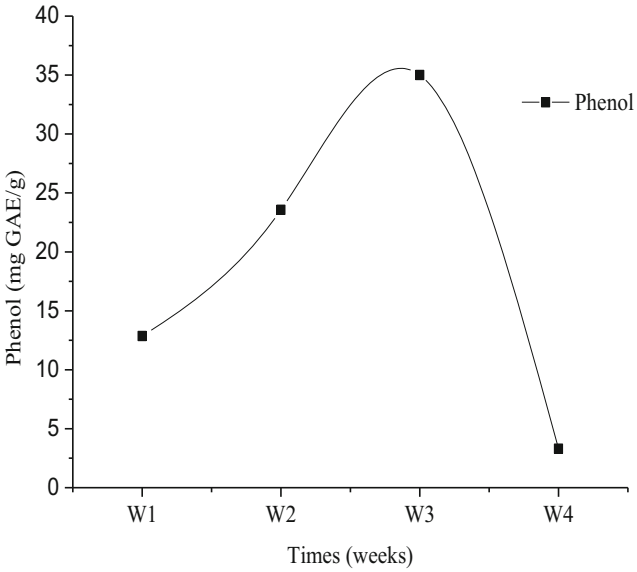

Fig. 7 Phenolic content in tomato fruits during spontaneous fermentation (W1–44: 4 weeks)

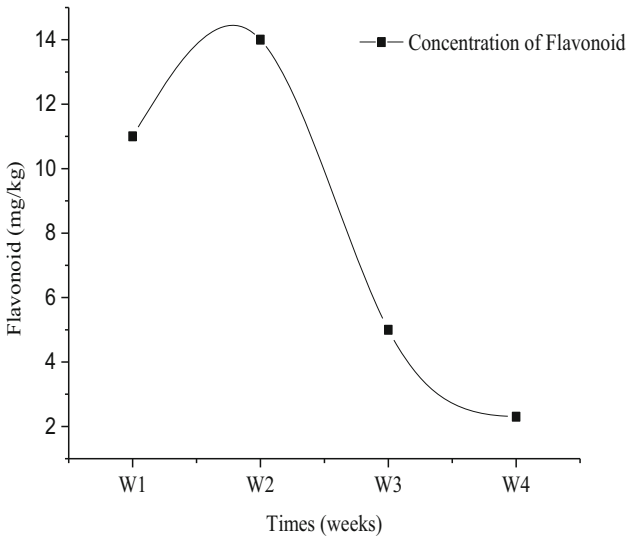

Fig. 6 The quantity of flavonoid produced during spontaneous fermentation of tomato fruit (W1–W4: 4 weeks)

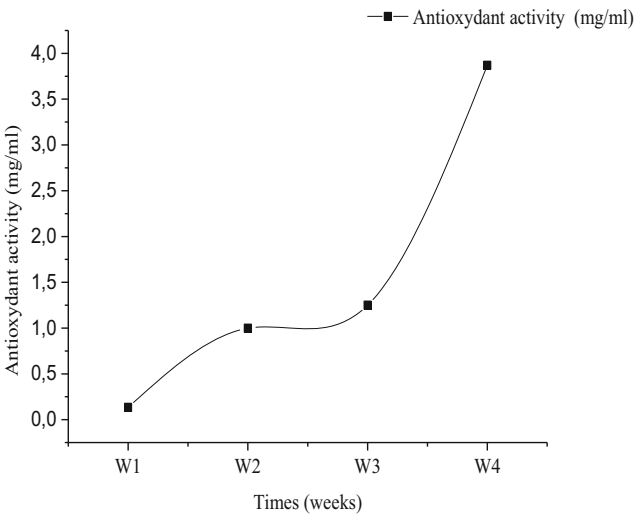

Fig. 8 Total antioxidant activity during the 4 weeks of spontaneous fermentation of tomato fruits (W1–W4: 4 weeks)

suggested the implication of several LAB metabolites, such as organic acid, bacteriocins, ethanol, and hydrogen peroxide.

Lycopene, flavonoid, and antioxidant compound determination

Lycopene, flavonoid, and total phenolic contents were investigated during spontaneous fermentation. The lycopene content estimated in tomato fruits during spontaneous fermentation increased progressively each week and reached 70 mg/kg at the last week (Fig. 5). The noted amount may derivate from different existing microorganisms, known as a potential source for bio-pigment production (Dufossé 2006). Flavonoid was also quantified on tomato fruits. During the first 2 weeks, flavonoid content was important (11 to 14 mg/kg), but decreased at the third and fourth week of fermentation reaching a low amount of 2.3 mg/kg at the last week (Fig. 6). As shown in Fig. 7, total phenol content increased at the beginning of the fermentation process and we recorded a concentration equal to 23 mg GAE/g, whereas it decreased at the last week to 3.3 mg GAE/g. The obtained results showed that the technological features were affected during the fermentation process (Javanmardi and Kubota 2006).

The antioxidant activity of tomato fruits was assessed during the spontaneous fermentation process. The results demonstrated that the antioxidant activity ranged from 0.134 to 3.87 mg GAE/g at the last week as demonstrated in the Fig. 8. LAB, which have proteolytic activity, release peptides that degrade phenolic compounds to simpler forms, thus stimulating the antioxidant activity. In concordance with Kachouri et al. (2015), the antioxidant activity increased in function of LAB growth on fruits of tomatoes.

In conclusion, this study provides an intriguing view of the microbial community evolution of tomato fruits during spontaneous fermentation process. Several LAB encountered during the spontaneous fermentation expressed diverse technological features. Selected LAB could be a potent candidate for starter culture formulation at food industry level.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

Ethical approval This article does not contain any studies with human participants or animals performed by any of the authors.

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