POST PRINT
https://www.sciencedirect.com/science/article/pii/S074000201930019X
DOI: http://doi.org/10.1016/j.fm.2019.103262
Food Microbiology 84 (2019) 103262

Exploitation of *Prunus mahaleb* fruit by fermentation with selected strains of *Lactobacillus plantarum* and *Saccharomyces cerevisiae*

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Abstract

The organoleptic attributes of *Prunus mahaleb,* a fruit representing a new source of bioactive compounds, are so pronounced that it can be consider non-edible. This study was designed to evaluate the acceptance of *P. mahaleb* fruits after fermentation with different *Saccharomyces cerevisiae* and *Lactobacillus plantarum* protechnological strains. Four different bacterial and one yeast strains, as single or mixed starter formulation, were used to inoculate an aqueous suspension of *P. mahaleb* fruits. The fermented fruits and fermentation broths were subjected to physico-chemical characterization and the organoleptic properties of both samples were also assessed by a hedonic panel. The obtained results indicated that all the employed strains were able to grow and to ferment the matrix. However, the mixed starter FG69 + Li180-7 (*L. plantarum*/*S. cerevisiae)* had the best impact on sensory characteristics of *P. mahaleb* fruit and fermented medium. The adopted protocol allowed us to attain edible fruits and a new fermented non-dairy drink with valuable probiotic health-promoting properties. In our knowledge, this is the first study concerning the exploitation of *P. mahaleb* fruits. This investigation confirmed the potential of yeasts and lactic acid bacteria co-inoculation in the design of starter tailored for this kind of food applications.

Keywords: *Prunus mahaleb* fruit; Fermentation; Deacidification; *Lactobacillus plantarum*; *Saccharomyces cerevisiae*; Probiotic beverage

1. Introduction

Prunus mahaleb L. is a tree belonging to the*Rosaceae* family, native to Europe and Western Asia and it is widely grown in warm climate of Central and South-Europe, Central Asia and Northern Africa. The tree exists in wild but it is also cultivated as cherry rootstock due to its tolerance to drought, poor soils and hot summers. Moreover mahaleb seed powder is used as flavoring agent for industrial baking and are known for its use in traditional medicine and cosmetics (Gökşen and Ekiz, 2016; Mariod et al., 2010).

P. mahaleb fruit is a small drupe with sour taste and black color when ripened. Even though sour taste of mahaleb fruits hamper their fresh consumption, recent investigations suggest that they could represent a new source of bioactive substances potentially useful for food and nutraceutical industries (Blando et al., 2016; Ieri et al., 2012). Indeed, recent papers demonstrated antioxidant capacity and biological activities of *P. mahaleb* fruits by isolating and identifying nine different polyphenolic molecules i.e. four anthocyanins, coumaric acid derivatives and flavonols (Blando et al., 2016; Ieri et al., 2012). Studies on biological activities of *P. mahaleb*fruit hydrophilic extracts suggest that they possess antibacterial, antioxidant, anti-proliferative, anti-inflammatory and anti-mutagenic activities on *in vitro* cell culture assays (Gerardi et al., 2016, 2015; Özçelik et al., 2012).

However, the application of a novel technological approach is requested to obtain edible *P. mahaleb* fruits.

The polystyrene resins could offer an efficient approach to obtain the debittering of this fruit (Bazrafkan et al., 2017), however this process is associated with environmental and economic costs. On the opposite, enhancing the acceptance by means of fermentation process, carried out by using yeast strains and lactic acid bacteria(LAB), represents a sustainable method, and it is in charge for other many positive features, such as flavor improvement, enhanced texture and extended shelf life (Randazzo et al., 2016). In addition, LAB can influence the amounts of valuable molecules, such as antioxidants, exopolysaccharide and vitamin, during fruit fermentation (Di Cagno et al., 2013). Finally, fermented fruits and vegetables can add supplementary health-endorsing attributes as they can introduce probiotic microorganisms in the human digestive tract (Saad et al., 2013).

As a result, yeast- and LAB-promoted fermentations can be identified as the easiest and valuable biotechnological approach to maintain and/or improve the safety and the quality of both vegetables and fruits and of the derived foods and beverages (Arena et al., 2016; Berenguer et al., 2016; Caputo et al., 2012; Di Cagno et al., 2013).

Recently (Yu et al., 2015), used a *Lactobacillus fermentum* strain to promote the fermentation of *Prunus mume* fruits demonstrating the possibility to process fermented fruits into dried food or sauces and to use the resulting fermentation broth for the production of an innovative probiotic beverage.

Considering the consumers' interest for fresh foods and beverages denoted by relevant probiotic, nutritional and functional properties (De Candia et al., 2017), this investigation was aimed to assess the chemical and organoleptic properties of *P. mahaleb* fruits fermented using different strains of *Saccharomyces cerevisiae* and *L. plantarum*.Experiments were carried out in order to obtain edible fruits, in terms of sensory acceptance, as well as to produce a novel non-dairy fermented beverage with valuable health-promoting properties by a single fermentation process. To the best of our knowledge, this is the first study concerning the possible commercial exploitation of *P. mahaleb* fruits and of the fermented drink from them derived.

2. Materials and methods

2.1. Microbial strains

Four *L. plantarum* and one *S. cerevisiae* strains were used as starters for *P. mahaleb* fruit fermentations, already characterized for their optimal fermentative properties resepectively for wine (Capozzi et al., 2017) and table olive (Bleve et al., 2014) production. The *L. plantarum* FG61, FG68, FG69 strains were isolated from grape must (Spano et al., 2002) and they are deposited in the collection of the Industrial Microbiology Laboratory (University of Foggia).The *L. plantarum* CI180-11 and *S. cerevisiae* LI180-7 strains were isolated from table olives (Bleve et al., 2014) and they can be purchased from Kron Morelli s.r.l. (Alfianello, Brescia, Italy). Before fermentation experiments, bacterial and yeast strains were stored at −80 °C in a 25% glycerol solution (Hay et al., 1994), were grown for 24–48 h at 30 °C in MRS broth (De Man, Rogosa and Sharpe, VWR International, Leuven, Belgium) and YPD broth (1% yeast extract, 2% peptone, 2% glucose), respectively.

2.2. Plant material

Organic *P. mahaleb* fruits were purchased from a nursery located in Sammichele di Bari (Bari, Italy), at fully ripe stage; leaves, unripe fruits and other un-necessary vegetable material were removed, and the fruits were stored at −20 °C until fermentation.

2.3. Standards and reagents

All solvents and reagents employed were of HPLC grade and supplied by Sigma-Aldrich (St. Louis, MO, USA); all microbial media were purchased from Biolife Italia (Milan, Italy).

2.4. Fermentation of *P. mahaleb* fruits

The *P. mahaleb* fruits were submitted to a fermentation process, inspired by previous protocols that we developed for the production of table olives (Tufariello et al., 2015) as following (Fig. 1S). Thawed fruits were blanched by dipping them in boiling water for 1 min and moved to cold sterile water for additional 1 min. After blanching, *P. mahaleb* fruits (100 g) were distributed into sterilized glass flasks containing sterile distilled water (500 mL) under aseptic conditions.
Each of the strain was inoculated at an initial concentration of 10^6colony forming units per millilitre (CFU/mL) independently by single or mixed (bacteria + yeast) culture; a not-inoculated blanched control was set up. Two separate fermentation assays were carried out in duplicate, the former during the harvest season 2015 and the latter during the harvest season 2016. So results are the average values ± SD of four replications. The drupes were incubated at 25 °C for 20 days; fruits and fermentation broth were sampled regularly; part of these samples were immediately evaluated for microbial populations whereas remaining amounts were stored at −20 °C for further chemical assays.

2.5. Microbial analyses

The viable count of yeasts was performed on WLN agar medium (*Wallerstein Nutrient medium*, Oxoid, Basingstoke, England) according to Tristezza et al. (2016). Lactic acid bacteria were enumerated onto MRS agar supplemented with 0.05 g/L nystatin, after 48–72 h incubation at 30 °C under anaerobic conditions, whereas *Enterobacteriaceae* onto VRBG (*Violet Red Bile Glucose*, Biolife, Milan, Italy) at 37 °C for 18–24 h. Before the sampling of the microbial population, the flask containing the fermenting fruits were shaked for 5 min on a orbital shaker in order to allow the breaking down of bacterial aggregates. Each count was performed in duplicate and results were expressed as log CFU/mL.

2.6. Sugar and organic acid extraction from fermented P. mahaleb fruits

Sugars and organic acids were extracted from fermented *P. mahaleb*as described by Gerardi et al. (2015). Briefly, fruits were pitted and blended (Ika, Germany) in the presence of liquid nitrogen. Sugar and organic acid were extracted from powdered fruits using water as solvent (5 mL/1 g). The mixture was centrifuged (Allegra X-15 R-Beckman Coulter, USA) at 4000×*g* for 30 min. The

extract was filtered on 0.45 μm nylon filters (LLG-Labware, Germany) and stored at −20 °C until analysis. Each extraction was performed in duplicate.

2.7. Determination of pH, organic acids and sugars

The values of pH of fermentation broth and fermented fruit extracts were measured by a properly calibrated pH meter (Eutech Instrument, The Netherlands). Organic acids and sugars were determined in both fermentation broth and fermented fruit extract by High Performance Liquid Chromatography (HPLC) using the Agilent-1100 apparatus (Agilent, USA), equipped with an UV-visible detector monitored at 210 nm for the analysis of organic acids and a refractive index detector (RID) for sugar analysis (De Benedictis et al., 2011). Sugars and organic acids were simultaneously identified onto an Aminex HPX-87H column (Bio-Rad, 300 × 7.8 mm, 9 μm) kept at 55 °C. The eluent used was 0.045 N H_2SO_4 with 6% acetonitrile (v/v) with a flow of 0.3 mL min^{-1} (Castellari et al., 2000). The identification of both sugars and organic acids were ascertained by comparison of their retention times and UV-Vis spectra with those of authentic standards. Quantification of individual sugars and organic acids were performed directly by ChemStation software (Agilent) using a five-point regression curve ($r^2 \geq 0{,}99$) on the basis of authentic standards. Acetic acid was quantified both in fermentation broth and fermented fruit extract using an enzymatic kit (Biogamma, Italia). The enzymatic kit protocol was modified to be performed using a 96 well microplate (Corning, USA) and a microplate reader *Infinite M200* (Tecan, Switzerland).

2.8. Evaluation of microbial survival to simulated gastro-intestinal conditions

In order to evaluate the probiotic potential of fermented food produced with *P. mahaleb* fruits, lactobacilli and yeast strains were grown into a new microbial broth having a chemical composition close to that found in fermentation broths (Table 1S). In this broth free amino acids and a source of vitamins were supplemented as it was found to be necessary to improve the growth of *L. plantarum*strains (data not shown). Microbial cells were then subjected to an *in vitro* digestion protocol specifically set up for the isolation of potential probiotic lactobacilli from milk samples (Baruzzi et al., 2011). In order to verify protocol feasibility, *L. johnsonii* NCC 533 and *L. helveticus* ATCC 15009^t strains, as well as *Saccharomyces boulardii* (isolated from CODEX, Zambon Italia, Milan, Italy) and *S. cerevisiae* DSM 70449^t were used as positive and negative control strains for probiotic lactobacilli and yeasts, respectively (Baruzzi et al., 2011).

2.9. Sensory analysis

Sensory analysis was carried out by seven non-trained panellists (between 23 and 60 years of age, three female and four male). At the end of both the fermentations, separately carried out during the harvest seasons 2015 and 2016, samples of fermentation broth and fermented *P. mahaleb* fruits were randomly coded and served at 20 °C together with table biscuits and mineral still water. Panellists were placed separately in rooms for objective evaluation of sensory attributes of the products. Samples were scored from 1 (lowest) to 5 (highest) for each sensory attributes. Taste was evaluated as sweetness, acidity and sourness; appearance referred to colour intensity for fermented fruits and to colour intensity and clearness for fermentation broth. Texture of the fermented fruits and viscosity of fermentation broth were evaluated during the tasting. Aromatic attributes were expressed as smell intensity and bad smell. Moreover, panellists expressed a hedonic judgement about the overall acceptability of the product (Di Cagno et al., 2010).

2.10. Statistical analysis

Significant differences among samples were determined by analysis of variance (ANOVA) (De Benedictis et al., 2013). Tukey's Honistly Least Significant Difference means comparison test was performed with a value of $P = 0.05$. To show the relationship between sensory data and chemical composition, partial least squares regression (PLSR) was carried out. Agglomerative Hierarchical Clustering (AHC) was performed using Ward's method and the dissimilarity was measured by Euclidean distance. The assigned clusters were then used to build the parallel coordinates plot of the classes' profiles. The statistical software packages used for these analyses were PAST 3.14 (Hammer et al., 2001) and XLSTAT 19.02 (Addinsoft, France).

3. Results

3.1. Microbial growth kinetics and acidification

L. plantarum strains FG61, FG68, FG69, inoculated as single culture at 6.0 log CFU/mL, reached an average cell density of respectively 9.5, 9.1 and 8.9 log CFU/mL in samples after 20 days of fermentation at 25 °C. In the case of CI180-11strain the higher concentration in viable cell count was found after 7 days of fermentation at 8.6 log CFU/mL (Fig. 1A). These viable cell concentrations were not observed when *L. plantarum* strains were co-inoculated with *S. cerevisiae.* Concerning the behaviour of the *S. cerevisiae* strain LI-180-7, when it was inoculated as single culture or in combination with *L. plantarum* FG61 strain, grew to 8.3.0 log CFU/mL after 4 days at 25 °C and then it decreased respectively to 6.0 log CFU/mL and 3.3 log CFU/mL at the end of fermentation (Fig. 1B). Even though viable cell count of *L. plantarum* at the 4th day of

fermentation did not show significant differences due to the presence of *S. cerevisiae* LI-180-7, the growth of FG61, FG68 and FG69 strains was slowed down from the day 8. At the end of fermentation, the FG61 and FG68 strains reached a concentration of about 6.0 log CFU/mL, whereas the FG69 showed a cell count corresponding to 7.2 log CFU/mL (Fig. 1A). The co-inoculated CI180-11 bacterial strain showed a higher value of cell numbers and precisely 8.8 log CFU/mL at the end of fermentation (Fig. 1A). Li-180-7 yeast strain inoculated together with FG68, FG69 and CI180-11 bacterium strains grew to about 7.0 log CFU/mL after 4 days of fermentation and then decreased to cell densities ranging from 5.0 to 2.5 log CFU/mL (Fig. 1B). The blanching step was found to be sufficient to hamper any visible microbial growth as showed by un-inoculated control samples as well as by the absence of colony development belonging to *Enterobacteriaceae* in all samples (data not showed). As concern changes in pH values, both broth and fruit samples inoculated with monocultures of different *L. plantarum* strains, as well with co-cultures bacteria + yeast, reduced pH from an initial value of about 4.5 to a value ranging from 4.2 to 3.5, depending on strains. On the contrary, no significant pH reduction was recorded for broths or fruits inoculated with *S. cerevisiae* LI180-7 as well as for control samples (Fig. 2S A-D).

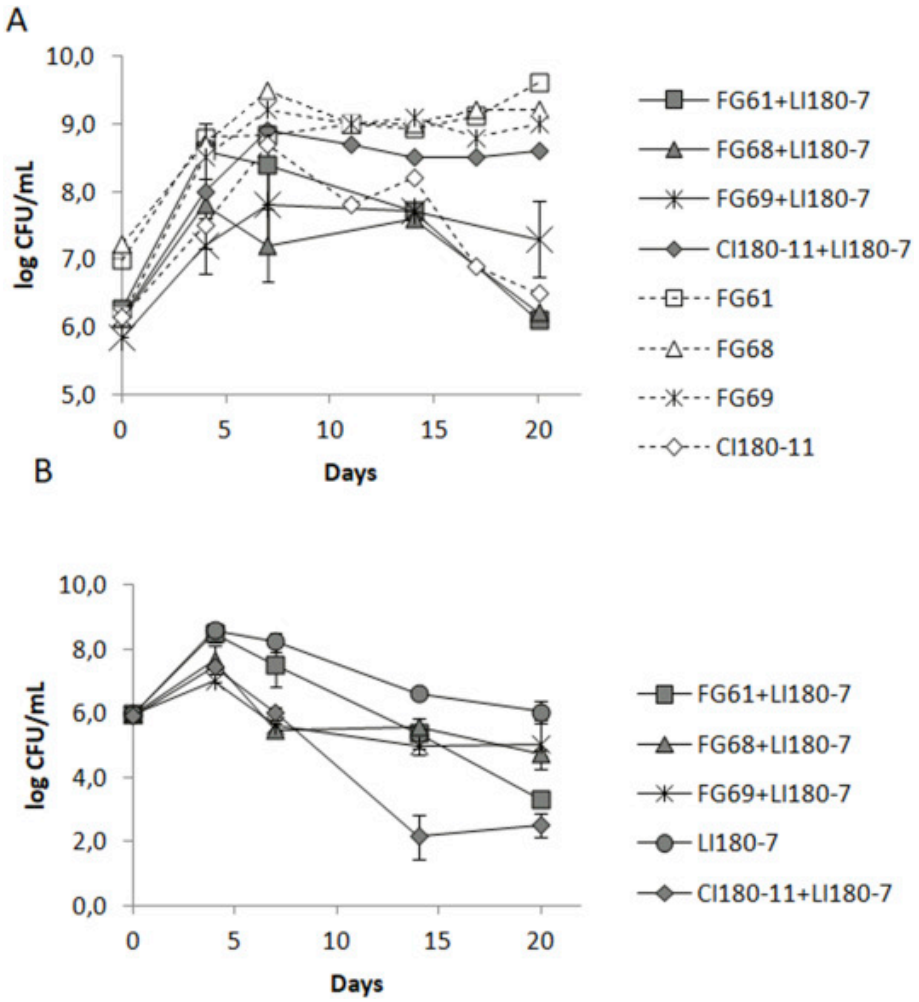

Fig. 1. Cell numbers (log CFU/mL) of *L. plantarum* bacteria in fermentation broth of *P. mahaleb* fermented by pure strains or by mixed starters (A). Cell numbers (log CFU/mL) of *S. cerevisiae* yeast strain LI180-7 in fermentation broth of *P. mahaleb* fermented by pure yeast strain or by mixed starters (B). Data are the means of two independent experiments carried out in duplicate± standard deviations (n = 4).

3.2. Sugars, organic acids, ethanol and glycerol analysis

The HPLC analysis performed in this study allowed the simultaneous identification and quantification of glucose, fructose, sorbitol, succinic acid, malic acid, lactic acid, ethanol and glycerol.

Glucose content in unfermented *P. mahaleb* fruits was 39.46 ± 5.76 mg/g FW (fresh weight), during incubation glucose diffused from fruit into the broth as demonstrated for control samples by the increase of glucose in fermentation broth up to 10.8 g/L and its decrease up to 8.3 mg/g FW in control fruits at the end of experiment (Fig. 2). Glucose trends in fermentation broths and fermented fruits inoculated with monoculture *L. plantarum*strains fermentation were similar to glucose trends in control fermentation broths and control fermented fruits (Fig. 2B–D). On the contrary, in all fermented samples inoculated with *S. cerevisiae*LI180-7, glucose diffused more efficiently in fermentation broths and was readily consumed (Fig. 2A–C) becoming undetectable already after

seven days of incubation. The mixed starter CI180-11 + Li180-7 was the most efficient in glucose fermentation (Fig. 2A–C).

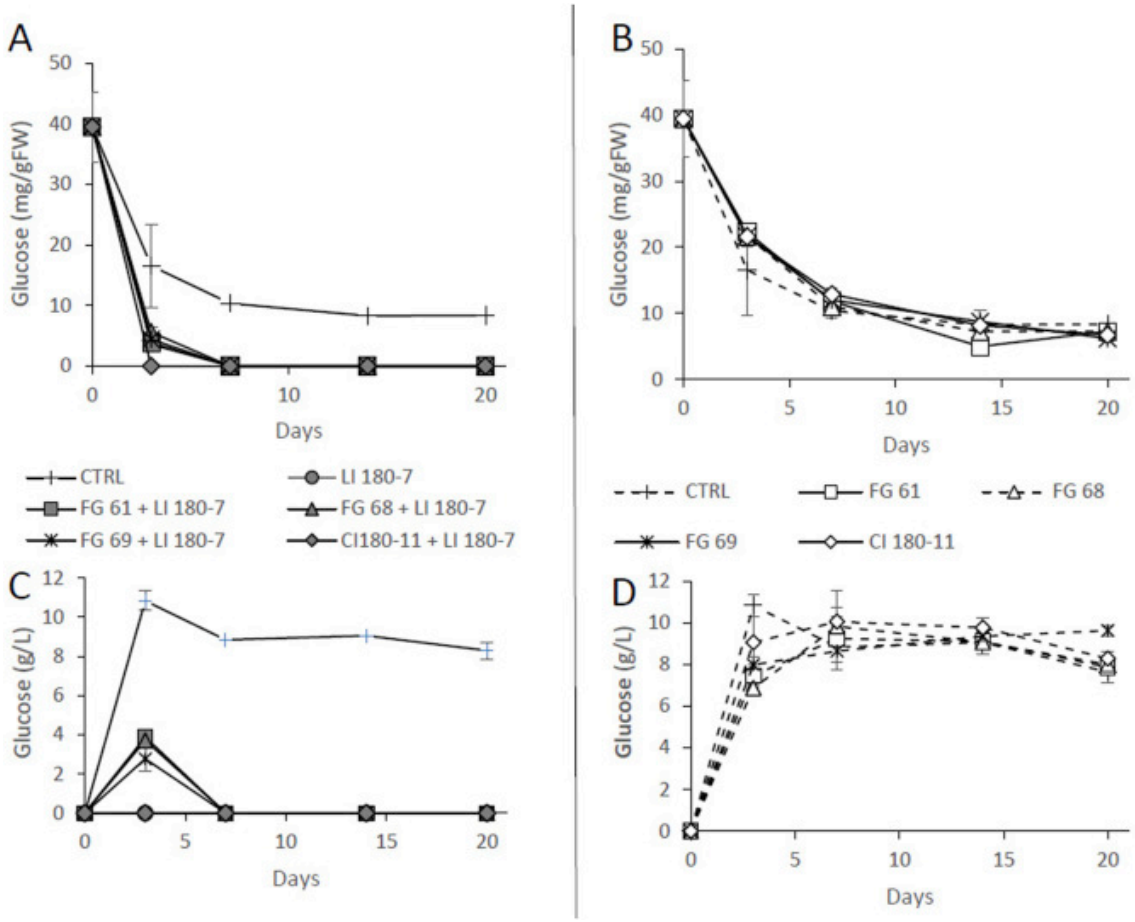

Fig. 2. Changes in glucose content in *P. mahaleb* fruits (A-B; mg/gFW) and fermentation broth (C-D; g/L) during fermentation with four *L. plantarum*strains and one *S. cerevisiae* strain inoculated as single starter or as bacterium + yeast mixed starter. Not-fermented control (CTRL). Data are the means of two independent experiments carried out in duplicate ± standard deviations (n = 4).

Fructose content in mahaleb fruits was initially found to be 37.236 ± 9.193 mg/g FW; in not fermented control fruits, fructose diffused from the fruits into the broth and its content increased up to about 9 g/L after three days and then slowly decreased, meanwhile a decrease of fructose was measured, up to 7 mg/g FW, in the fruits (Fig. 3S). The fruits fermented by single *L. plantarum*strains showed a fructose content trend similar to the control both in fermentation broth and in fermented fruits (Fig. 3S B-D). As already found in the case of glucose, fructose diffused from fruit to fermentation broth and here was readily utilized only by *S. cerevisiae*LI180-7 or by mixed bacterial - yeast starters; the most efficient starters was CI180-11 + LI180-7 (Fig. 3S A-C).

Sorbitol content in *P. mahaleb* fruits at the beginning of fermentation was 33.310 ± 9.510 mg/g FW; sorbitol diffused quickly, but not completely, from the fruits to the fermentation broth in not

inoculated and inoculated samples, but, differently from glucose and fructose, sorbitol was not metabolized remaining stable at about 8 g/L in fermentation broth (Fig. 4S A-D).

Succinic acid and tartaric acid content in *P mahaleb* fruits at the beginning of fermentation was 1.30 ± 0.05 mg/g FW and 14.0 ± 0.14 mg/g FW respectively. After three days of fermentation, their concentration in fruits dropped to undetectable levels due to their diffusion into the broth where their concentration increased during three days and remained stable in all samples for 20 days of fermentation (data not shown).

Differently from succinic and tartaric acids, malic acid underwent some reduction due to microbial activity. Its concentration in the fruits was measured at 1.95 ± 0.08 mg/g FW and diffused from fruits to broth during fermentation (Fig. 3, Fig. 4A and B). The diffusion into the broth increased its concentration up to 1.54 ± 0.10 g/L already after three days remaining stable till the end of incubation (Fig. 3C and D). In fermentation broth samples inoculated with bacteria, as well as with mixed starters, malic acid was not detectable probably because it was readily utilized by *L. plantarum*(Fig. 3C and D). However, differences were observed with *S. cerevisiae*alone: after 7 days of fermentation, malic acid concentration in fermentation broth increased up to 3.55 ± 0.28 g/L (Fig. 3C). The diffusion of malic acid from fruit to fermentation broth induced by bacterial fermentation was corresponding to the decrease of malic acid in the fermented fruits that became not detectable after three days of fermentation, while in not fermented control and yeast fermented samples, malic acid content decreased but was still detectable till the end of fermentation (Fig. 3 A).

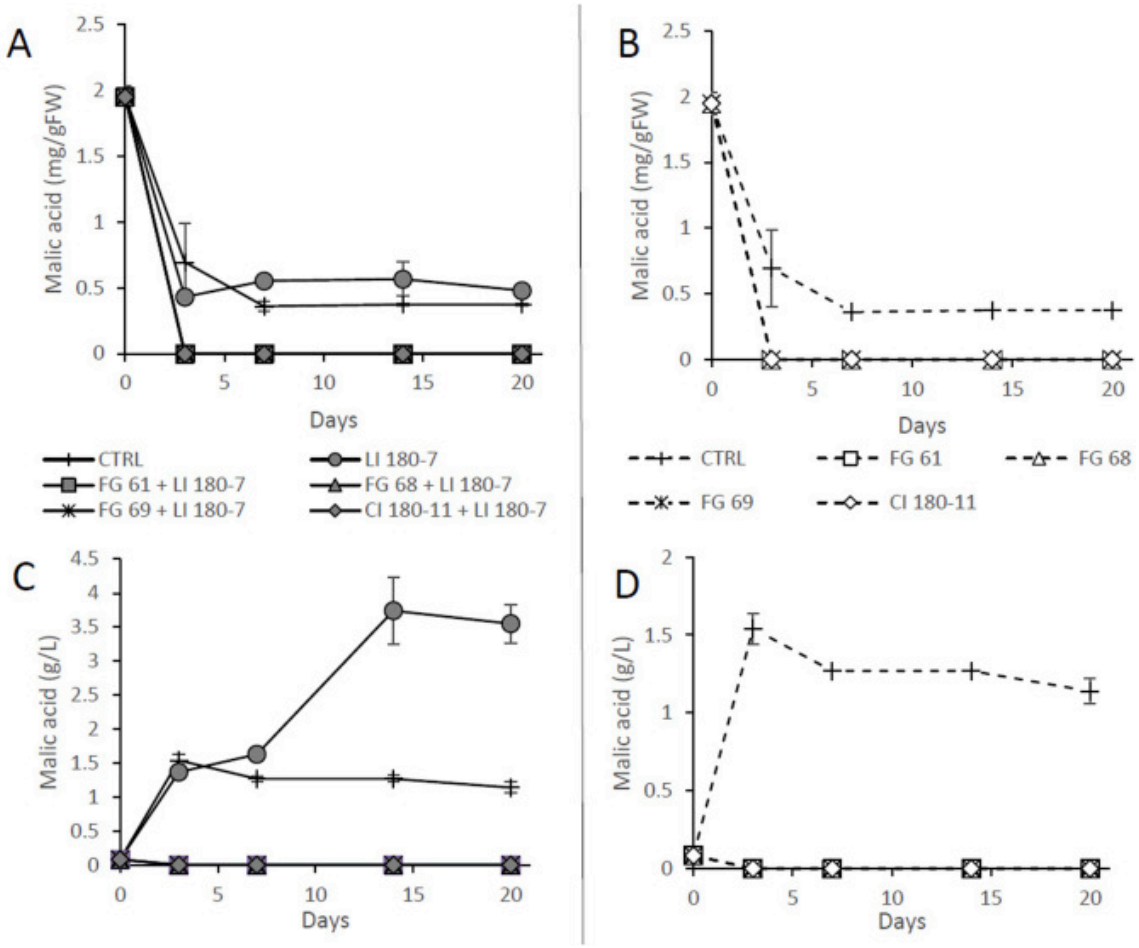

Fig. 3. Changes in malic acid content in *P. mahaleb* fruits (A-B; mg/gFW) and fermentation broth (C-D; g/L) during fermentation with four *L. plantarum*strains and one *S. cerevisiae* strain inoculated as single starter or as bacterium + yeast mixed starter. Not-fermented control (CTRL). Data are the means of two independent experiments carried out in duplicate ± standard deviations (n = 4).

Acetic acid increase was detectable only in fermentation broth inoculated by mixed starters; in control sample and in fermentation induced by pure yeast and bacterial starters, acetic acid content was very low and stable (Fig. 4A). The higher values of acetic acid (0.6 g/l) was measured utilizing FG61 + Li180-7 and CI180-11 + Li180-7 mixed starters (Fig. 4A).

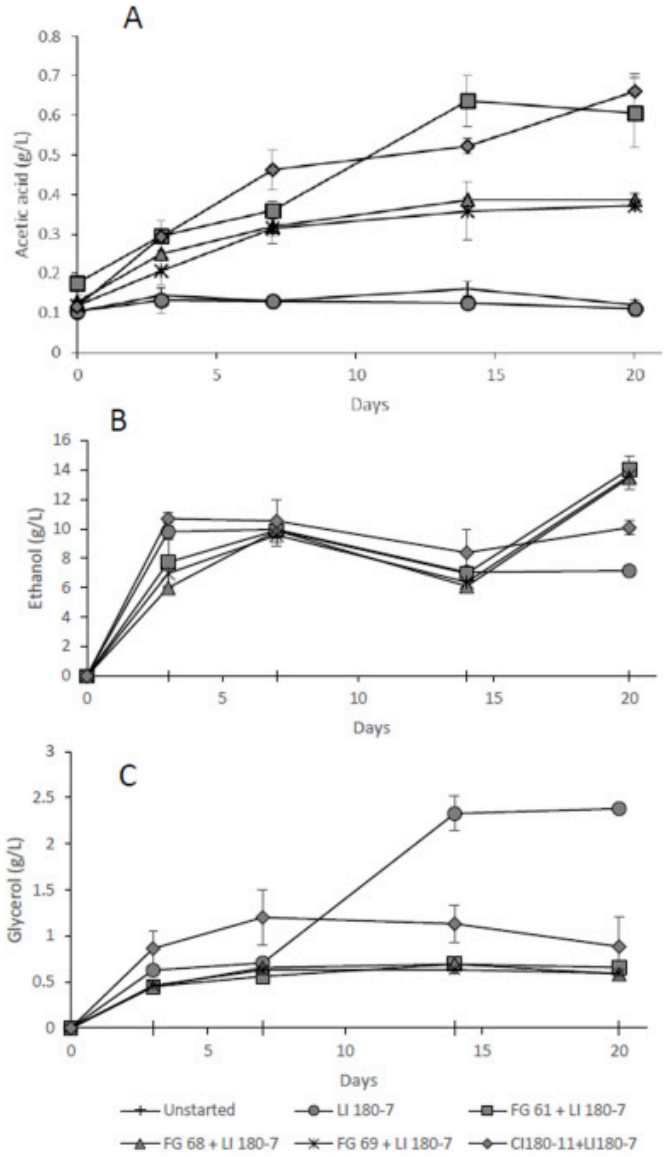

Fig. 4. Changes in acetic acid (A), ethanol (B) and glycerol (C) content (g/L) in fermentation broth during *P. mahaleb* fruits fermentation with four *L. plantarum* strains and one *S. cerevisiae* strain inoculated as single yeast starter or as bacterium + yeast mixed starter. Not-fermented control (CTRL). Data are the means of two independent experiments carried out in duplicate ± standard deviations (n = 4).

Ethanol was not detectable in broth of control sample and in fermentation induced by L. *plantarum* pure culture starters; it was identified and quantified in all fermentation broth samples inoculated with *S. cerevisiae*. Ethanol content increased very quickly within three days of fermentation, slowly till seven days of fermentation and then underwent a slight decrease up to 15 days of fermentation (Fig. 4B). Successively all samples fermented by mixed starters revealed an increase of ethanol content up to 14 g/L except CI180-11+LI-180-7 that showed a stable ethanol content of 10 g/L (Fig. 4B). It is interesting to note that at day 20 all co-cultures showed higher ethanol content than *S. cerevisiae* monoculture.

Glycerol was identified and quantified in the fermentation broth of fruits fermented by *S. cerevisiae* and by mixed starters and was not detectable in control sample and in fermentation induced by L. *plantarum* pure culture starters. Glycerol content increased up to 0.5 g/L for all

mixed starters except CI180-11+LI-180-7 that showed a higher content of glycerol (1.2 g/L) (Fig. 4C). After 7 days of fermentation, the glycerol content was stable till the end, but during the fermentation with pure yeast starter an increase was quantified from 7 to 14 days of fermentation up to 2.30 ± 0.19 g/L (Fig. 4C).

Lactic acid showed a steady increase in fermentation broth inoculated with *L. plantarum* strains reaching the highest concentration (about 12 g/L) after 20 days when pure bacterial starters, *L. plantarum*, were utilized, while in control and in *S. cerevisiae* fermented sample lactic acid was undetectable (Fig. 5A and B). Acetic acid, ethanol, glycerol and lactic acid were not detectable in the extracts of fermented fruits.

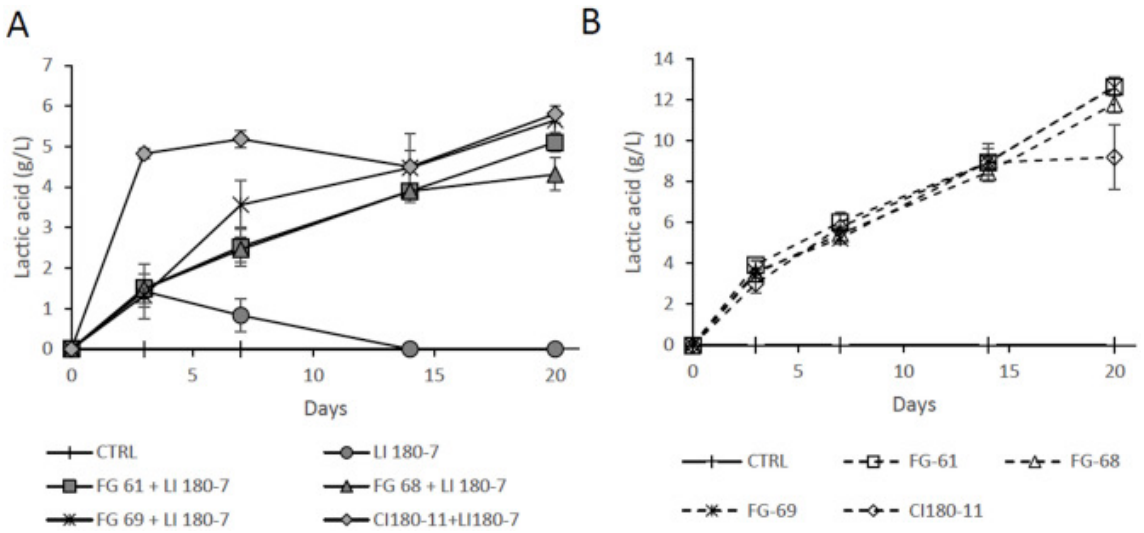

Fig. 5. Changes in lactic acid (A–B) content (g/L) in fermentation broth during *P. mahaleb* fruits fermentation with four *L. plantarum* strains and one *S. cerevisiae* strain inoculated as single starter or as bacterium + yeast mixed starter. Not-fermented control (CTRL). Data are the means of two independent experiments carried out in duplicate ± standard deviations (n = 4).

3.3. Survival to simulated gastro-intestinal conditions

Based on chemical characterization of fermented juice, a synthetic *P. mahaleb* broth was made up as described in Table 1S. Since this medium differs from fermented prunus juice itself, it cannot exactly represent the evolution of viability that would occur in the fermented juice. Nevertheless it is useful in comparing the resistance of *L. plantarum* and *S. cerevisiae* LI180-7 strains themselves to *in vitro* gastro intestinal digestion, after 24 h of incubation at 37 °C. This media allowed *L. plantarum* strains to grow (as found after about three days on fruit infusion in water) at an average concentration of 8.58 ± 0.20 log CFU/ml. The different ability found of fermentation broth to sustain the growth of the same *L. plantarum* strains after several days of incubation could be due to the time dependent release of nutrients from fruit to broth as described above. In the same broth, also probiotic positive and negative *Lactobacillus* strains grew well at an average concentration of 8.96 ± 0.38 log CFU/ml. After digestion steps all lactobacilli were still alive showing a reduction in

their viability of at about 5 magnitude orders. However, only the strains *L. plantarum* FG68 and *L. johnsonii* NCC 533 (probiotic strains) were still viable after further incubation of 24 h in MRS supplemented with bile salts.

The survival of *S. cerevisiae* LI180-7 at the end of the selection/enrichment digestion protocol was considered to be not reliable because, differently from lactobacilli, both positive (probiotic) and negative *Saccharomyces* strains, were still viable. Based on this result no evaluation related to probiotic features of co-colture was carried out.

3.4. Sensory analysis

The average ratings of the sensory attributes are shown in Table 1, for fermented broth, and Table 2, for fermented fruits. From the results of the one-way ANOVA using the Tukey HSD test on the descriptive data, only the descriptor *sourness* was found to be significantly different ($p < 0.05$) across samples, for both fermented broth (Table 1) and fermented fruit (Table 2) samples.

Table 1. Sensory characteristics and hedonic mean value of fermented broths given by judges.

Empty Cell	**LI180-7**	**FG69 + LI180-7**	**FG69**	**FG68 + LI180-7**	**FG68**	**FG61 + LI180-7**	**FG61**	**CI180-11 + LI180-7**	**CI180-11**	**Blank**
Sensory										
Clearness	1.71 ±0.76	1.86 ±1.07	2.14 ±1.07	1.86 ±1.07	2.14 ±1.07	2.29 ±0.95	2.29 ±1.11	2.00 ±0.82	2.29 ±1.11	2.43 ±1.27
Color intensity	3.71 ±0.95	3.71 ±0.49	3.86 ±1.07	3.57 ±0.79	3.86 ±0.90	3.71 ±1.11	4.00 ±1.15	4.14 ±0.90	3.71 ±1.11	4.29 ±0.95
Viscosity	2.14 ±1.07	2.43 ±0.53	2.29 ±0.95	2.50 ±0.55	2.57 ±0.53	2.57 ±0.79	2.43 ±0.98	2.43 ±0.79	2.50 ±0.84	2.29 ±1.25
Smell intensity	2.86 ±1.77	3.71 ±0.95	3.29 ±1.25	3.00 ±1.41	3.14 ±0.90	3.14 ±1.35	3.00 ±1.41	3.14 ±1.57	3.43 ±1.27	3.29 ±1.70
Acidic	3.00 ±2.00	2.86 ±1.07	4.17 ±0.98	2.29 ±1.38	3.57 ±1.40	2.57 ±1.40	3.57 ±1.62	2.71 ±1.11	3.71 ±1.38	2.57 ±1.51
Sweetness	1.00 ±0.00	1.57 ±0.79	1.00 ±0.00	1.71 ±0.76	1.29 ±0.76	1.43 ±0.53	1.14 ±0.38	1.57 ±0.53	1.00 ±0.00	1.14 ±0.38
Sourness	2.86 ±1.68^{ab}	2.86 ±1.07^{ab}	4.43 ±0.98^{a}	2.57 ±1.13^{ab}	4.14 ±0.90^{ab}	2.29 ±1.25^{b}	3.57 ±1.51^{ab}	2.86 ±0.69^{ab}	3.29 ±1.50^{ab}	2.57 ±1.13^{ab}
Hedonic										
Overall acceptability	2.14 ±0.90	2.43 ±0.53	2.71 ±1.11	2.29 ±0.76	2.71 ±0.95	2.57 ±0.98	2.29 ±0.95	2.71 ±0.95	2.29 ±0.95	2.57 ±1.13

Lactobacillus plantarum strains: FG61, FG68, FG69, CI180-11. *Saccharomyces cerevisiae* strain: LI 180–7. Blank: not inoculated. Standard deviation values (±) are indicated; different letters indicate significant differences ($p < 0.05$) among the row.

Table 2. Sensory characteristics and hedonic mean value of fermented fruitsgiven by judges.

Empty Cell	**LI180-7**	**FG69 + LI 180-7**	**FG69**	**FG68 + LI 180-7**	**FG68**	**FG61 + LI 180-7**	**FG61**	**CI180-11 + LI 180-7**	**CI180-11**	**Blank**
Sensory										
Texture	2.43 ±1.27	2.14 ±0.90	2.86 ±0.69	2.43 ±1.27	2.83 ±1.17	2.57 ±0.98	2.50 ±0.84	2.80 ±0.84	2.86 ±0.90	2.43 ±1.27
Colour intensity	3.00 ±1.15	3.00 ±1.00	3.57 ±1.40	3.43 ±1.13	3.86 ±1.35	3.86 ±1.35	4.00 ±0.82	3.57 ±1.27	3.71 ±1.38	3.43 ±1.13
Smell intensity	2.57 ±1.13	3.14 ±1.07	3.00 ±1.15	3.29 ±1.38	2.57 ±0.79	3.00 ±0.82	2.86 ±0.69	3.00 ±1.15	2.86 ±0.90	2.14 ±0.69
Bad smell	1.00 ±0.00	1.14 ±0.38	1.14 ±0.38	1.14 ±0.38	1.29 ±0.49	1.14 ±0.38	1.43 ±0.79	1.14 ±0.38	1.00 ±0.00	1.71 ±0.95
Acidic	2.14 ±1.21	2.43 ±1.27	2.29 ±1.11	1.43 ±0.53	2.86 ±1.21	1.57 ±0.79	3.00 ±1.29	1.57 ±0.79	2.71 ±1.70	2.29 ±1.70
Sweetness	1.14 ±0.38	1.29 ±0.49	1.86 ±0.90	1.57 ±0.79	1.17 ±0.41	1.43 ±0.79	1.29 ±0.49	1.71 ±0.95	1.14 ±0.38	1.00 ±0.00
Sourness	2.29 ±1.50^{ab}	2.43 ±1.13^{ab}	2.14 ±1.21^{ab}	1.71 ±0.76^{ab}	3.00 ±1.41^{ab}	1.86 ±1.07^{ab}	3.29 ±1.11^{a}	1.29 ±0.49^{b}	3.29 ±1.11^{a}	2.86 ±1.21^{ab}
Hedonic										
Overall acceptability	2.43 ±1.13	2.57 ±0.98	2.67 ±0.82	2.86 ±0.69	2.83 ±1.33	3.14 ±0.69	2.33 ±0.82	3.14 ±0.69	2.71 ±0.95	2.00 ±1.00

Lactobacillus plantarum strains: FG61, FG68, FG69, CI180-11. *Saccharomyces cerevisiae* strain: LI 180–7. Blank: not inoculated. Standard deviation values (±) are indicated; different letters indicate significant differences ($p < 0.05$) among the row.

A PLS regression of the hedonic judgements *Y* and the product characteristics *X* is presented in Fig. 6; the type of inoculum is also shown. Samples of fermented broth on the upper-right side of the

biplot (Fig. 6A) are those inoculated with the single strains of *L. plantarum* and they are highly rated in *sourness*, *acidity* and *clearness*, and also have high concentrations of fructose, glucose and lactic acid. Three out of seven judges prefer these products. The co-inoculated samples are located on the lower-right side of the biplot (Fig. 6A). They was preferred by four panellists and showed higher rates on the sensory attributes *sweetness*, *viscosity* and *smell intensity*, positively associated with ethanol and acetic acid. The sample inoculated with only *S. cerevisiae*, on the lower-left side in Fig. 6A, was not characterized by any sensory attribute but has high concentrations of malic acid and glycerol and higher pH value. The not-inoculated control is related to the *colour intensity* attribute and to succinic acid. None of the judges preferred these two samples.

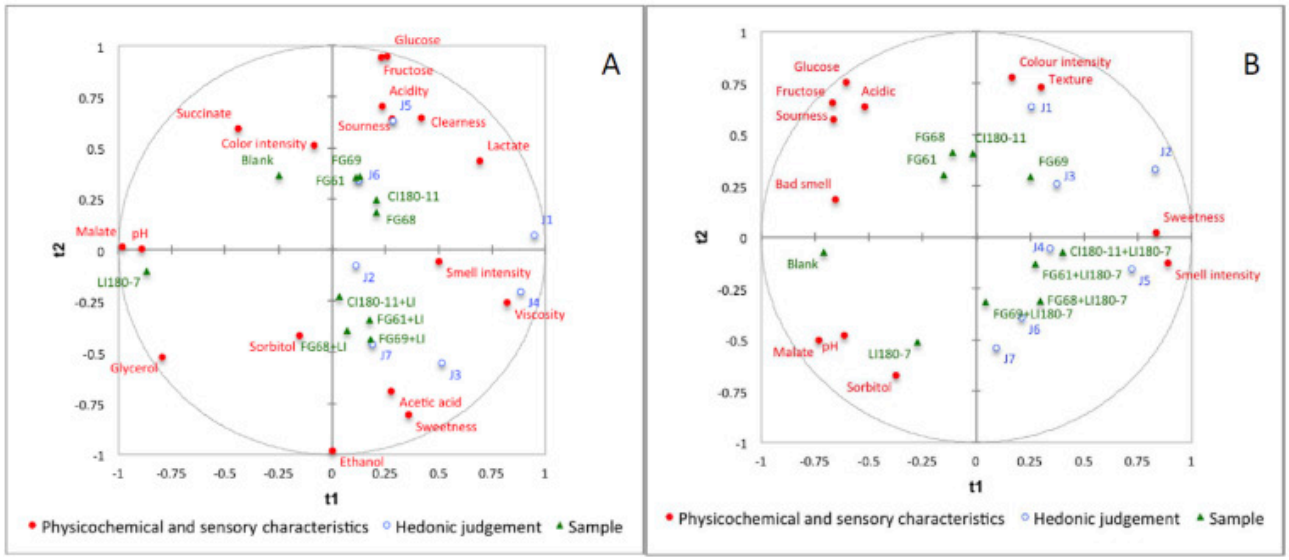

Fig. 6. Correlation circle of the samples, the mean values of physico-chemical and sensory characteristics and the hedonic judgement (overall acceptability score) given by each judge, with the two first PLS components. (A) fermented broth; (B) fermented fruits. *L. plantarum* strains: FG61, FG68, FG69, CI180-11. *Saccharomycescerevisiae* strain: LI 180–7. Blank: not inoculated.

Concerning fermented fruits, samples inoculated with the single strains of *L. plantarum*, on the upper side of the correlation circle (Fig. 6B), are related along the *t2* axis with residual sugars and the attributes *acidic*, *sourness*, *colour intensity* and *texture*, whereas fruits inoculated with the strain FG69, on the upper-right side of the plot, is more correlated along the *t1* axis with the attribute *sweetness* and it was preferred by three judges. On the lower-right side of the plot (Fig. 6B), fruits inoculated with the mixed starters FG69 + LI180-7 cannot be related to product characteristics but two judges seemed to prefer this sample; while samples inoculated with FG61 + LI180-7, FG68 + LI180-7 and CI180-11 + LI180-7 are characterized by *sweetness* and *smell intensity* and two judges preferred these products. The fruits inoculated with only *S. cerevisiae*, on the lower-left side in Fig. 6B were not characterized by any sensory attribute

but they have high concentrations of malic acid and sorbitol and higher pH value. The blank control is related along *t1* axis to the *bad smell, acidic* and *sourness* attributes with high concentrations of glucose and fructose. None of the panellists preferred these two samples.

4. Discussion

Fermentation is known to improve preservation, safety, nutritional and functional properties and acceptability of many vegetable foods (Chen et al., 2017; Di Cagno et al., 2013; Feng et al., 2017). However, its application to new plant species requires preliminary studies for the selection of fermentative microbial starters that best adapt to plant matrices.

The spontaneous fermentation of *P. mahaleb* fruits as suitable process to obtained derived food products was immediately excluded after some preliminary assays due to high final concentration of acetic acid (ten times the acetic acid concentration of controlled fermentation) and the growth of contaminating *Enterobacteriaceae* (data not shown). These results are in agreement with previous works assessing that spontaneous fermentation has a risk of failure both for safety and sensory changes of the fermented product (Di Cagno et al., 2008; Urbonaviciene et al., 2015), confirming the possible problems for human health associated with the exploitation of spontaneous fermentations in the food sector (Capozzi et al., 2017). The protocol for fruit fermentation included a blanching step that was effective in inactivate the microbial population of *P. mahaleb* fruits as no yeast, moulds or bacteria were detected in not inoculated control fermentation. Successively four allochthonous strains of *L. plantarum*, isolated from different plant materials (see Materials and methods) where inoculated as single cultures or as mixed starters with a strain of *S. cerevisiae* isolated from table olives. As presented in Fig. 1A, the *L. plantarum* strains utilized in this fermentation study, showed a good adaptation to plant material environment having abundant polyphenolic compounds (Blando et al., 2016) confirming already reported results for several commercial/allochthonous bacterial strains during vegetable fermentation (Di Cagno et al., 2008; Gardner et al., 2001).

In fact, there was a constant microbial growth for all the bacterial starter during the 20 days of fermentation at 25 °C. A general trend comparable to that reported by Yu and collaborators (2015) in a similar experiment conducted on fruits of *Prunus mume* using a *L. fermentum* strain.

Cell density of bacterium monoculture strain starter was higher than mixed starter cell density after 20 days of fermentation except for *L. plantarum* CI-180-11 strains. Growth of the yeast is also affected by the presence of lactobacilli as yeast cell concentration of pure starter is higher than mixed starters at end of fermentation. These results confirm the statement that lactobacilli and yeast are antagonistic to each other (Thomas et al., 2001) due to competition for nutrient and/or inhibitory

substances such as ethanol produced by the yeast (Narendranath et al., 1997). However, with the exeption of the CI180-11, the *L. plantarum* viable cell count was found to be lower than 10^7 CFU/mL that is the cell density delivering about 10^9viable cells into the intestine by a an approximate amount of 100 g of probiotic food per day (Tripathi and Giri, 2014).

The decrease in pH values for both fermentation broth samples and fermented fruits reflects the diffusion of succinic and tartaric acidfrom fruits to medium as well as the production of lactic acid and acetic acid during malolactic and alcoholic fermentations. Almost the same change of pH was reported during fermentation of vegetables (carrots, cabbages, beets, French beans, etc.) (Di Cagno et al., 2008; Gardner et al., 2001) and fruits (sweet cherry, pineapple and pomegranate) (Di Cagno et al., 2011a; Di Cagno et al., 2011; Filannino et al., 2013). All starters we used, except LI180-7, achieve a reduction of pH to values (pH 3 – pH 4) low enough to inhibit the growth of the major part undesirable microorganisms (Doyle and Buchanan, 2013). Sugars and acids flowed from the fruit into the liquid as early as the first 3 days of fermentation. Glucose and fructose were completely metabolized only in samples started with yeast, in which the accumulation of ethanol and glycerol, metabolites normally produced during alcoholic fermentation, were also observed. It is interesting to note that in this study the *S. cerevisiae* CI180-11 showed better efficiency in simple sugarutilization than *L. plantarum* strains; a similar results was also reported for *Candida zemplinina* CBS 9494 when co-cultured with *Acetobacter syzygii* LMG 21419 in glucose supplemented minimal medium to understand the role of carbon sources in developing table grape sour rot (Pinto et al., 2019).

Ethanol, the main product of alcoholic fermentation, contributes to enhance the sensory characteristics of fermented products and has a preservative function as it inhibits the development of unwanted microorganisms (Swiegers et al., 2005). The low ethanol content (Fig. 4B) found in the fermenting liquid of *P. mahaleb* fruits (less than 2%) is in line with the requests of consumers who are more oriented towards the consumption of fermented beverages with lower alcohol content (Giaramida et al., 2016). Glycerol (Fig. 4C) is an important by-product of alcoholic fermentation, important in determining sweetness and giving roundness and body to fermented beverages (Arroyo-López et al., 2010). A very low acetic acid production (Fig. 4A) has been detected only in medium of *P. mahaleb* fruits fermented by mixed starters. It is known (Thomas et al., 2001) that yeast and facultative heterofermentative lactobacilli, utilized as pure culture starters, produce very low amount of acetic acid, but its amount increases when yeasts are added to fermentation medium after bacteria. During the fermentation of *P. mahaleb* fruits, the *L. plantarum* strains behaved as homofermentative LAB. During fruit fermentation, the higher content of acetic acid (about 0.6 mg/L) was measured at the end of fermentation using mixed starters CI180-11 + Li180-7 and

FG61 + Li180-7. Both these two mixed starters showed at the end of fermentation the higher decrease in yeast cell density, that was probably due to the content of acetic acid (>0.5 g/L) (Thomas et al., 2001).

As reported by Gerardi et al. (2015), *P. mahaleb* fruit extract contains tartaric acid, succinic acid and malic acid that are associated with sour and bitter taste of fruits; during fermentation succinic and tartaric acid diffused into the fermentation broth with the same trend, in the presence of all starters and in the control experiment. After three days of fermentation, succinic and tartaric acid reached the maximal value in the fermentation broth and were undetectable in the fruits but any starters have not metabolized these acids. Malic acid also diffused from the fruits to the fermentation broth, but in the presence of *L. plantarum* strains, it is immediately fermented to lactic acid and after three days of fermentation is undetectable both in fruits and in fermentation broth (Fig. 3). The malolactic fermentation carried out by *L. plantarum* strains has been described for many plant matrices, especially in conditions of extreme acidity (Di Cagno et al., 2011a). The increase in malic acid content during fruit fermentation with *S. cerevisiae* (Fig. 3C) is in agreement with previous studies demonstrating that L-malic acid is a product of fermentation by yeast (Dakin, 1924) and sustained by three different inter-connected biochemical pathways (Zelle et al., 2008). Malolactic fermentation also had a positive effect on the organoleptic characteristics of fermented products, as the lactic acid imparts a softer taste and replaces the acidic and astringent acidic acid while simultaneously improving the microbiological stability of fermented products (Torriani and Felis, 2014).

Higher lactic acid content in fermentation broth of *L. plantarum* pure culture compared to fermentation induced by mixed starter (Fig. 5) is related to higher cell density of bacterium pure culture after 20 days of fermentation than bacterium cell density during mixed starter fermentation (Fig. 1A).

The results of fermented broth and fruit samples sensory analysis indicated a positive effect of fermentation carried out by the starter mixture, which simultaneously take advantage of alcoholic and malolactic fermentation, placing these samples in the right lower part of the sensory correlation plot (Fig. 6).

In the present study, we used partial least square analysis to study the relationship between sensory properties and product characteristics. PLS is commonly used to relate instrumental and sensory data (Chambers and Koppel, 2013). Several researches had used this technique for create maps of correlation between sensory descriptors (*Y*-variables) and instrumental data (*X*-variables) of orange juice (Tenenhaus et al., 2005), wine (Vilanova et al., 2012), fresh strawberries (Schulbach et al., 2004) and blackcurrant drinks (Piggott et al., 1993). In this study, the PLS denotes that some

chemical compounds are related to particular sensory attributes rather than others (Fig. 6). There is a positive association of *sourness*and *acidic* attributes with glucose and fructose, which are negatively associated with the attribute *sweetness*. This kind of correlation was previously reported in orange juice (Tenenhaus et al., 2005). The attribute *colour intensity* is positively related with the lactic acid and negatively associated with malic acid probably because the lactic fermentation could have a preservative effect on the colour (Di Cagno et al., 2011b). There is a clear effect of the type of inoculum on the sensory rating of the samples. The lactic fermentation positively affected the sensory attributes since all judges prefer products where the *L. plantarum* strains were inoculated instead of the not inoculated control or the sample with only the yeast strain. Similar evidence was observed with fermented pomegranate juices (Filannino et al., 2013) and fermented smoothies (Di Cagno et al., 2011a). However, *L. plantarum* strains did not had the same impact on sensory characteristics showing the couple FG69 + Li180-7 as the most suitable for *P. mahaleb* fruit fermentation.

As concerning probiotic potential of *L. plantarum* strains, and beverage produced from fruits, it was found that, they were all able to survive to simulated gastric and pancreatic digestion, confirming the interest in *L. plantarum* species (Gheziel et al., 2018) and in vegetables as a potential source of probiotic bacteria (Peres et al., 2012). It is worthy of note that these strains were able to survive without no protective effect of food matrix as instead found when probiotic strains included in milk based dairies were subjected to *in vitro* simulated gastro intestinal digestion protocols (Champagne et al., 2015; Klu and Chen, 2015; Madureira et al., 2011; Verruck et al., 2015).

Additionally, the amino acids of peptones of yeast extract in the simulated medium (Table 1S) could potentially enhance the survival of bacteria to the acid stress of the gastric condition (Feehily and Karatzas, 2013; Wu et al., 2014).

Since different *L. plantarum* strains were employed for fruit fermentation and beverage production, the static gastrointestinal *in vitro* simulation protocol described by Baruzzi et al. (2011) applied only in order to compare the best survival of *L. plantarum* strains grown in their broth.

However differences were found the further resistance to bile salts that was found only for the strain *L. plantarum* FG68 allowing to guess that the beverage here reported can be considered as an innovative fermented foods of plant origin that now are being increasingly employed as probiotic vectors (Soccol et al., 2010). Even though no assays were carried out in this study, the occurrence of *S. cerevisiae* in fermentation broth could improve gastrointestinal resistance of *L. plantarum* strains due to already reported mechanisms of co-aggregation and biofilm formation between cells of these microbial species (Furukawa et al., 2011).

However, in order to have preliminary information about potential survival of *L. plantarum* FG68 and *S. cerevisiae* LI180-7 when assumed with fermented *P. mahaleb* fruits the application of standardised static *in vitro* digestion protocols suitable for foods need to be employed (Brodkorb et al., 2019; Minekus et al., 2014).

Thus, we can conclude that sourness of *P. mahaleb* fruits can be efficiently reduced by fermenting them thank a new starter co-culture made up with *L. plantarum* and *S. cerevisiae* producing new kinds of functional and probiotic foods and beverages. Our results indicated that the fermentation of *P. mahaleb* fruits produced a nondairy beverage denoted with potential healthy probiotic properties. Indeed, fermentation with the LAB strain *L. plantarum*CCMA 0743 as have been already demonstrated to confer potential health benefits to fermented product (Şanlier et al., 2017). Moreover, the quick and ample acidification and production of organic acids enhance the safety of the produced beverage, avoiding the presence of undesired pathogen (Freire et al., 2017). To the best of our knowledge the co-culture *L. plantarum* - *S. cerevisiae* was never applied to improve fruit quality and, contemporaneously, to produce a potential probiotic vegetable-based beverage. In addition, the results here reported open the possibility of a sustainable exploitation of *P. mahaleb,* a plant resistant to biotic stress and unfavorable climatic and soil conditions, representing a possible resource for specific regions, and, more generally, to face to the new trends associated with global climate changes. Finally, we underline one of the emerging exploitations of the fermentation processes: mitigate sensory attributes improving the acceptance of vegetable matrices rich in bioactive and with a 'functional' interest. Studies are in progress on the effect of lactic and alcoholic fermentation on antioxidant activity and functional molecule content of fermented *P. mahaleb* fruits and fermentation broth, and on the protective role yeast cells during gastrointestinal digestion of bacterial probiotic cells, in order to better characterize a probiotic drink source of bioactive compounds.

Acknowledgements

The authors wish to thank Mr. Giovanni Colella and Mr. Leone D'Amico for their valuable technical assistance and Prof. H. Smith for proofreading and providing valuable linguistic advice.

Appendix A. Supplementary data

The following are the Supplementary data to this article:

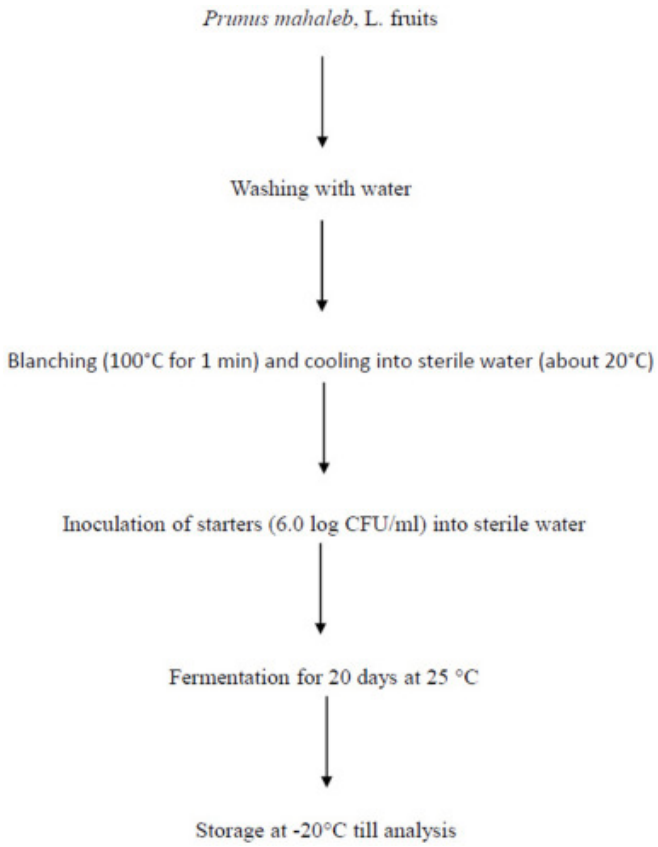

Fig. s1. Protocol for fermentation of *Prunus mahaleb* L. fruits.

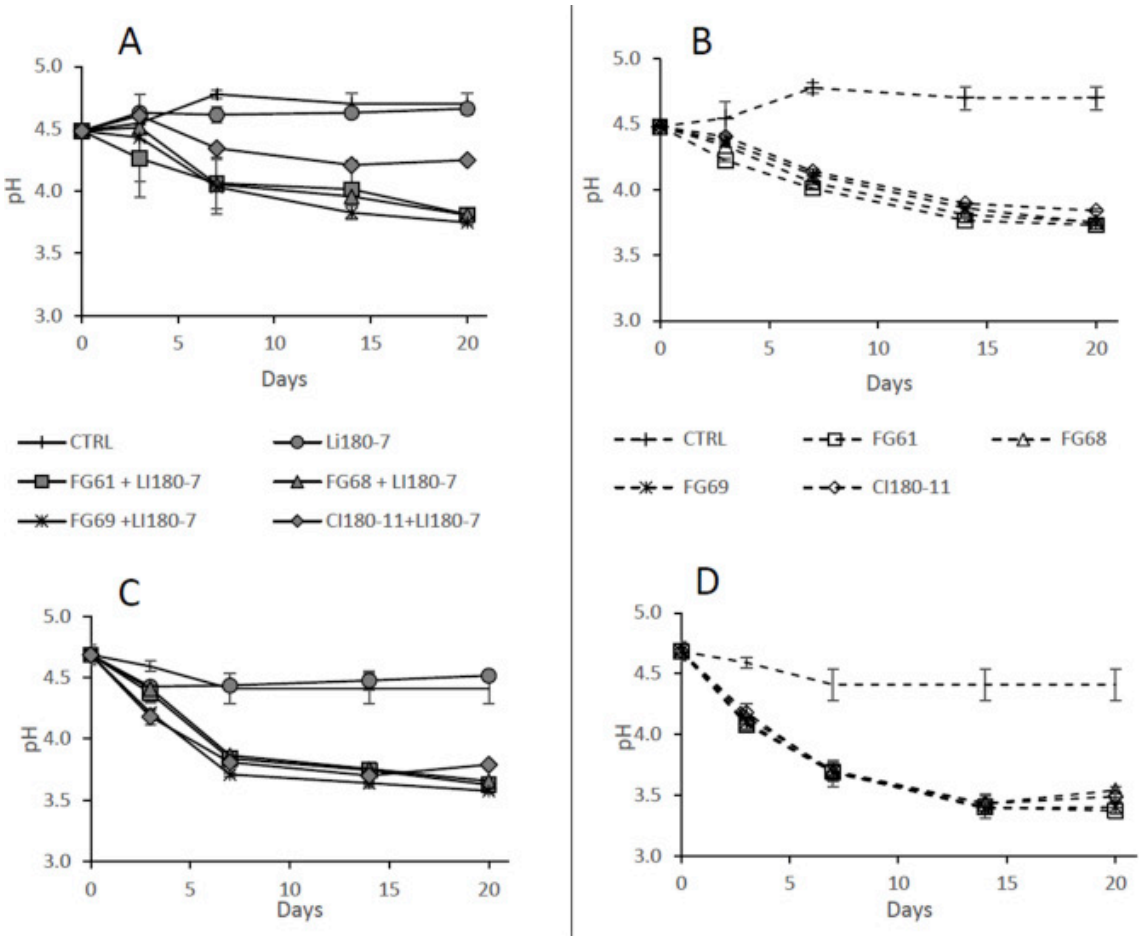

Fig. s2. Changes in pH in *P. mahaleb* fruits (A-B) and fermentation broth (C-D) during fermentation with four *L. plantarum* strains and one *S. cerevisiae* strain inoculated as single starter or as bacterium + yeast mixed starter. Not-fermented control (CTRL). Data are the means of two independent experiments carried out in duplicate ± standard deviations (n = 4).

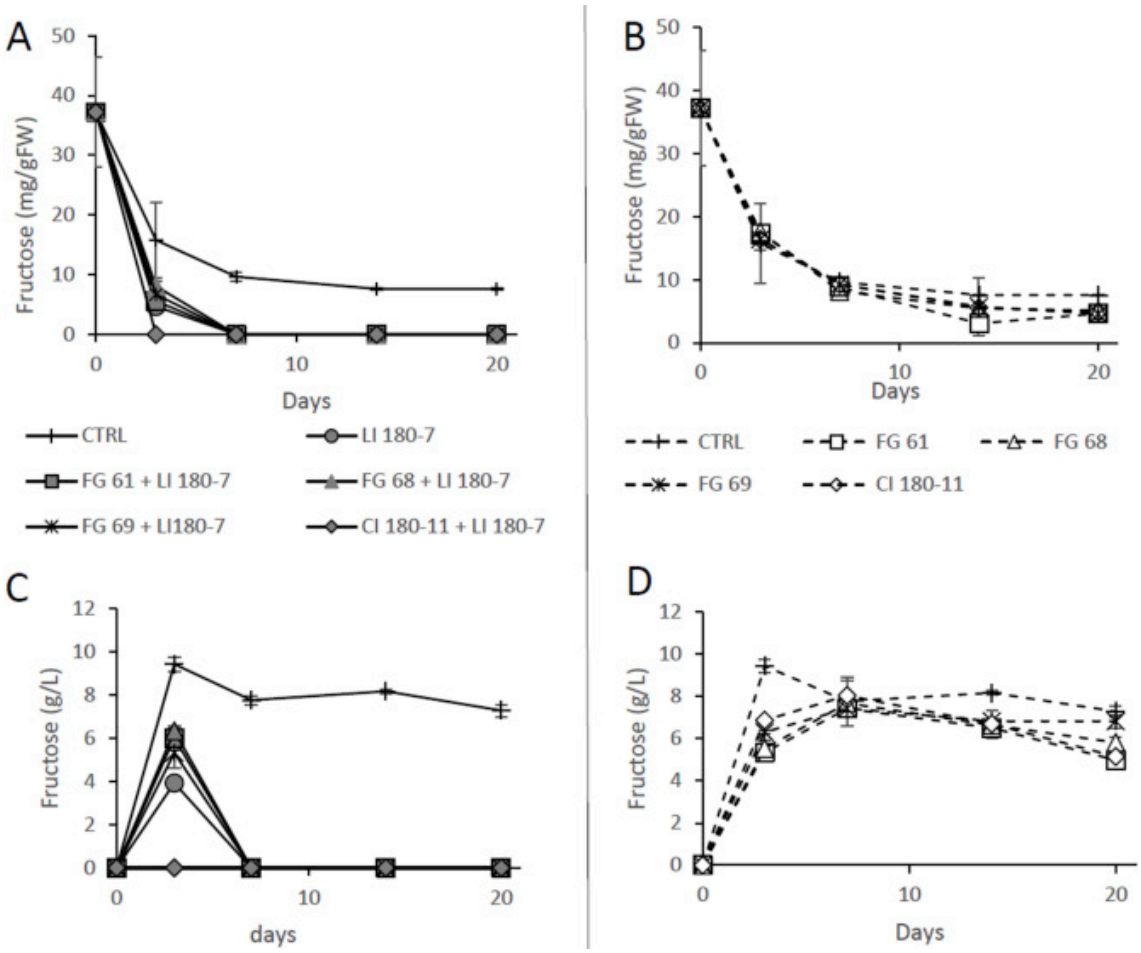

Fig. s3. Changes in fructose content in *P. mahaleb* fruits (A-B; mg/gFW) and fermentation broth (C-D; g/L) during fermentation with four *L. plantarum* strains and one *S. cerevisiae* strain inoculated as single starter or as bacterium + yeast mixed starter. Not-fermented control (CTRL). Data are the means of two independent experiments carried out in duplicate ± standard deviations (n = 4).

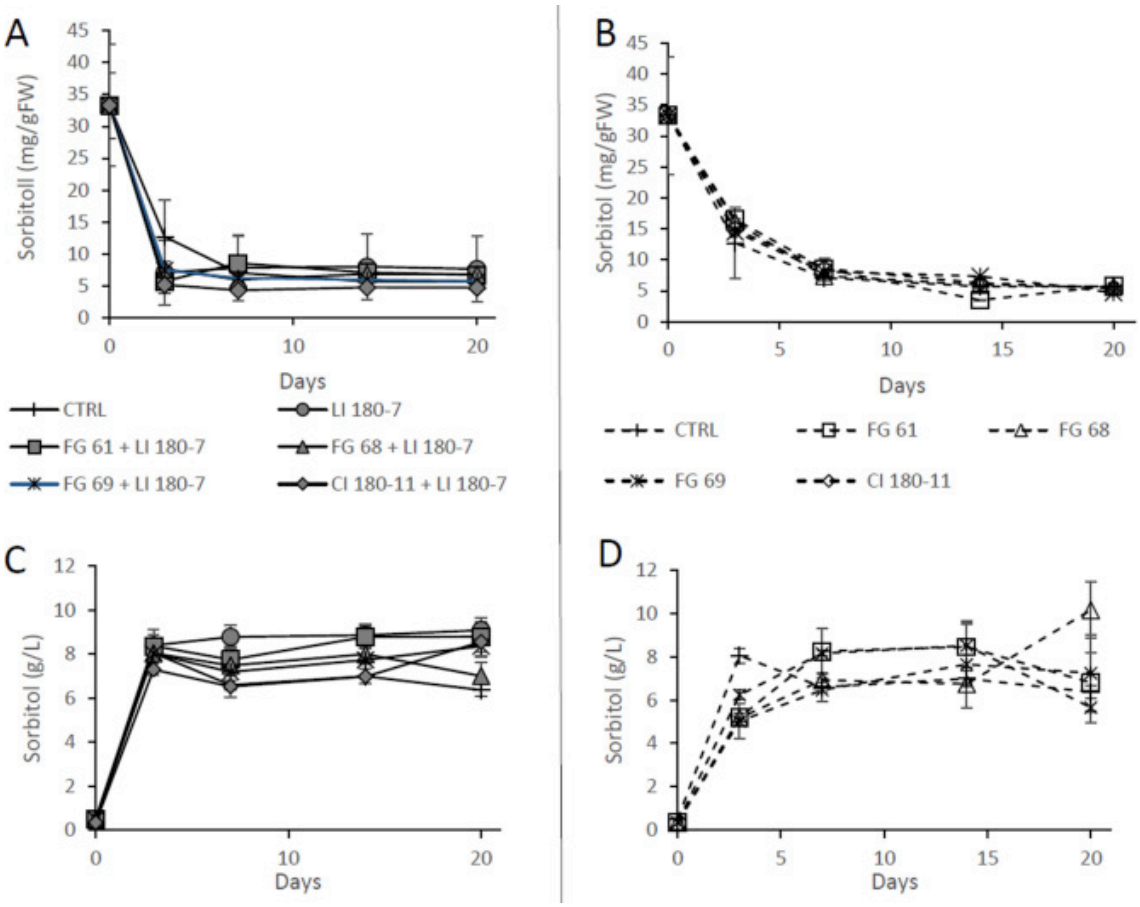

Fig. s4. Changes in sorbitol content in *P. mahaleb* fruits (A-B; mg/gFW) and fermentation broth (C-D; g/L) during fermentation with four *L. plantarum* strains and one *S. cerevisiae* strain inoculated as single starter or as bacterium + yeast mixed starter. Not-fermented control (CTRL). Data are the means of two independent experiments carried out in duplicate ± standard deviations (n = 4).

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