Ann Microbiol (2016) 66:1377–1386
DOI 10.1007/s13213-016-1225-3

ORIGINAL ARTICLE

Antiradical and antimicrobial properties of fermented red chicory (*Cichorium intybus* L.) by-products

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Received: 10 March 2015 / Accepted: 30 May 2016 / Published online: 10 June 2016
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Abstract Discarded leaves of red chicory (Radicchio "Rosso di Chioggia" IGP) were fermented with one *Saccharomyces* yeast and four lactic acid bacteria chosen on the basis of their ability to grow on plant material without any need of supplements. Antiradical and antimicrobial activities of the resulting products were assessed. Among the strains tested, *Lactobacillus plantarum* and *L. hilgardii* gave the best performances and also provided fermented substrates with antiradical and antimicrobial activities. In particular the latter compounds were found only in fermented samples, confirming that the choice of appropriate microorganisms for fermentation could be useful when the aim is to target specific functional foods starting from by-products or waste material.

Keywords *Cichorium intybus* · By-products · Fermentation · Antioxidant/antiradical activity · Antimicrobial activity · Functional food

Introduction

The genus *Cichorium* includes four wild species (*C. bottae*, *C. spinosum*, *C. calvum*, and *C. pumilum*) and two widely cultivated ones, namely *C. intybus* (chicory) and *C. endivia* (lettuce). Chicory is a Mediterranean vegetable that is also widespread throughout Europe, Western Asia and North America, including several varieties with leaves varying in color from white to dark red. Some cultivars are seasonal, while other are produced continuously throughout the year, such as the red chicory "Radicchio rosso di Chioggia. Commercial production of colored radicchio varieties involves a laborious procedure that includes the removal of many outer leaves from heads, with the aim of keeping the inside that are better developed and characteristic of the product. This procedure generates large amounts of by-products, the disposal of which represents a relevant environmental problem, not the least due to the presence of phenolic compounds (Arvanitoyannis and Kassaveti 2007).

Historically, chicory was used by the ancient Egyptians as a medicinal plant, and different parts of this plant are still utilized in folk medicines to treat various symptoms (Street et al. 2013). In the food industry chicory is considered to be a prebiotic because it is rich in minerals and inulin. Studies conducted to date have revealed the antioxidant potentialities of this vegetable (Innocenti et al. 2005; Rossetto et al. 2005; Koukounaras and Siomos 2010; Ferioli et al. 2015; Finotti et al. 2015) which are linked to its high content of phenolic compounds. Nevertheless, as yet chicory has not been shown to possess antimicrobial activity and, to our knowledge no study has investigated the ability of lactic acid bacteria to grow in this vegetable medium, possibly increasing its beneficial properties.

Fermentation by lactic acid bacteria (LAB) can be considered a simple and valuable method to preserve or even improve the safety, sensory and nutritional aspects of food products. Numerous LAB strains are routinely used as starter cultures in the dairy, meat and bakery industries, but a much more limited number have been used for fermentation of vegetables,

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among which *Lactobacillus plantarum* is the LAB starter most frequently used commercially (Ruiz-Barba et al. 1994; Leroy and De Vuyst 2004). This species is commonly part of the autochthonous microbiota of raw vegetables and together with *Lactobacillus hilgardii*, *Oenococcus oeni*, *Lactobacillus brevis* and *Leuconostoc mesenteroides* is frequently isolated from grape musts and other plant material (Moreno-Arribas et al. 2003; Rodas et al. 2005).

In the study reported here we tested several microorganisms for fermenting "Radicchio Rosso di Chioggia" leaves to assess the production of potential by-products with antiradical and antimicrobial activities.

Materials and methods

Strains and growth conditions

Strains used for fermentations and bacteria used as indicators of antimicrobial activity are listed in Table 1. Autochthonous strains of *L. hilgardii* and *L. plantarum* were isolated from plant material (Maragkoudakis et al. 2013).

Lactobacillus delbrueckii subsp. *bulgaricus*, *L. hilgardii* and *L. plantarum* were grown in de Man, Rogosa and Sharp medium (MRS; Oxoid Ltd., Basingstoke, UK), *Streptococcus thermophilus* in M17 medium (Oxoid), *Staphylococcus xylosus* in Corynebacterium Broth (Oxoid), *Listeria innocua* and *Escherichia coli* in Brain Heart Infusion (BHI; Oxoid) and *Bacillus amyloliquefaciens*, *B. subtilis* and *Pseudomonas fluorescens* in Nutrient Broth (NB; Oxoid). *L. delbrueckii* subsp. *bulgaricus*, *St. thermophilus* and *E. coli* were incubated at 37 °C; the other strains were incubated at 30 °C. *Saccharomyces cerevisiae* was grown in Yeast Mould Broth (YMB; Oxoid) at 30 °C for 24 h.

For preparation of inocula 8 ml of each culture at the stationary phase (approx. 10^8 CFU/ml) was centrifuged for 5 min at 5000 *g* to remove the supernatant and then resuspended in the same volume of saline solution (0.9 % NaCl w/v). For each cell suspension proper dilutions (approx. 1:1000) were performed in order to standardize the inoculum. Plate counts were performed to confirm the inoculum size.

Determination of autochthonous microbiota of chicory was performed by plate count on Plate Count Agar (PCA) medium (Oxoid) after 24 h of incubation at 30 °C.

Fermentation of red chicory leaves

Fresh heads of Radicchio "Rosso di Chioggia" were purchased from the local market and washed thoroughly with tap water. A 200-g sample of leaves was then mixed with 0.9 % NaCl at a ratio of 1:1 (w/v) and homogenized for 2 min with a blender. Aliquots of 30 g were rapidly transferred to 50-ml sterile Falcon tubes, inoculated with the appropriate microorganism to a final cell density of about 10^5 CFU/ml of homogenate and incubated at 30 °C for 48 h. A tube without inoculum was also incubated as control. All tests were performed in triplicate.

The samples were pasturized by immersing the Falcon tubes containing the inoculum and microorganism in a water bath at 72 °C for 15 min, followed by cooling at room temperature.

The pH values were measured after 0, 4, 24 and 48 h by immersing the pH-meter electrode directly into the tubes.

Freeze-drying of culture supernatants

When the pH of the fermenting cultures reached a steady value, the tubes were centrifuged at 5000 *g* for 15 min, and each supernatant was transferred into a new sterile Falcon tube and immediately freeze-dried. The powder was stored at −80 °C for later use. For the experiments, the powder was dissolved in sterile water at a final concentration of 200 mg/ml.

Antimicrobial activity assay

The well diffusion technique (Giacomini et al. 2000; Drosinos et al. 2007) was used to evaluate antimicrobial activity against the indicator bacterial strains reported in Table 1. The freeze-dried powders were used at final concentration of 4 mg/ml. With this technique, the formation of inhibition haloes surrounding the wells indicates the presence of activity, where the diameter of the haloes is related to the amount of inhibitory substance.

The proteinaceous nature of the inhibitory substance was checked by treating the extract with proteinase K (1 mg/ml; Sigma-Aldrich Corp., St. Louis, MO) before use and then looking for absence of inhibitory activity on the culture plates.

Determination of total polyphenol content and antioxidant activity

Total polyphenol content was measured using the Folin–Ciocalteau method (Singleton et al. 1999), with the concentrations expressed as gallic acid equivalents (GAE; Escarpa and Gonzales 2001) based on comparison against the gallic acid calibration curve (R^2 0.9049).

The DPPH (2,2-diphenyl-1-picrylhydrazyl; Sigma-Aldrich Ltd.) assay was used to determine the antiradical activity of the extracts, according to Miliauskas et al. (2004). Radical scavenging activity was calculated by the formula:

$$\% \text{ of inhibition} = (A_B – A_A)/A_B \times 100$$

Table 1 Bacterial strains used in the study

Strain	Source
Strains used for fermentations	
Lactobacillus delbrueckii subsp. *bulgaricus* DSM 20081^T	DSMZ (*)
Lactobacillus hilgardii DSM 20176^T	DSMZ
Lactobacillus hilgardii TGMMRS06	Maragkoudakis et al. 2013
Lactobacillus hilgardii TGMMRS08	Maragkoudakis et al. 2013
Lactobacillus hilgardii TGMMRS11	Maragkoudakis et al. 2013
Lactobacillus hilgardii TGMMRS12	Maragkoudakis et al. 2013
Lactobacillus hilgardii TGMMRS13	Maragkoudakis et al. 2013
Lactobacillus hilgardii TGMMRS25	Maragkoudakis et al. 2013
Lactobacillus hilgardii TGMMRS27	Maragkoudakis et al. 2013
Lactobacillus hilgardii TGMMRS35	Maragkoudakis et al. 2013
Lactobacillus hilgardii TGMMRS41	Maragkoudakis et al. 2013
Lactobacillus plantarum DSM 20174^T	DSMZ
Lactobacillus plantarum T30PCP06	Maragkoudakis et al. 2013
Lactobacillus plantarum T30PCP07	Maragkoudakis et al. 2013
Lactobacillus plantarum T30PCP14	Maragkoudakis et al. 2013
Lactobacillus plantarum T30PCP05	Maragkoudakis et al. 2013
Lactobacillus plantarum T30PCP01	Maragkoudakis et al. 2013
Lactobacillus plantarum T4MPCP71	Maragkoudakis et al. 2013
Lactobacillus plantarum T4MPCP69	Maragkoudakis et al. 2013
Lactobacillus plantarum T4MPCP77	Maragkoudakis et al. 2013
Lactobacillus plantarum T4MPCP78	Maragkoudakis et al. 2013
Lactobacillus plantarum T4MPCP73	Maragkoudakis et al. 2013
Saccharomyces cerevisiae DSM 70449^T	DSMZ
Streptococcus thermophilus DSM 20617^T	DSMZ
Strains used as indicators	
Bacillus amyloliquefaciens DSM 7^T	DSMZ
Bacillus subtilis DSM 10^T	DSMZ
Escherichia coli DSM 30083^T	DSMZ
Listeria innocua DSM 20649^T	DSMZ
Pseudomonas fluorescens DSM 50090^T	DSMZ
Staphylococcus xylosus DSM 20266^T	DSMZ

DSMZ, Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH

where A_B represents the absorption of blank sample ($t = 0$ min) and A_A is the absorption of the tested extract solution ($t = 15$ min).

Ascorbic acid at different concentrations was used as the control. All experiments were performed in triplicate.

Determination of phenolic acids and glucose

Phenolic acids were determined according to Rossetto et al. (2005) using a high-performance liquid chromatography (HPLC;Thermo-Finnigan, San Jose, CA) equipped with a photodiode array detector (UV 6000LP). All samples were filtered on a 0.45-μm cartridge and directly injected. The phenolic acids were separated using an LC-18 Suppelco-sil column (Sigma-Aldrich Ltd.) at 25 °C with a flow rate of 1.5 ml/min under isocratic conditions . The mobile phase consisted of an aqueous solution containing 1.5 % acetic acid and 18 % *n*-butanol. Phenolic acid standards were passed through the column both singly and in mixture. The spectrum of each standard was recorded and stored in the HPLC library data. Each reported value was the average of three repeated analyses. The internal and external standards included to identify the molecules on the basis of their UV spectrum and retention time were those reported by Lante et al. (2011). In brief, we compared spectral data of sample peaks with those obtained for the phenolic acids standard to confirm the reliability of the identification since matches between

the spectra of the samples and those of their standards showed a ≥90 % match.

D-Glucose content of chicory homogenates was determined by HPLC as reported by Zuleta and Sambucetti (2001). The HPLC system and conditions were as follows: an Aminex HPX-87C (Bio-Rad, Hercules, CA) anion-exchange column, deionized water at 85 °C as the mobile phase and a flux rate of 0.6 mL/min. To identify the sugars present in the samples, we used reference solutions of mono- and disaccharides.

Results and discussion

Fermentation of chicory homogenates

Red chicory homogenates were used as the growth substrate for type strains of one yeast (*S. cerevisiae* DSM 70449^T) and four LAB (*L. delbrueckii* subsp. *bulgaricus* DSM 20081^T, *L. hilgardii* DSM 20176^T, *L. plantarum* DSM 20174^T and *St. thermophilus* DSM 20617^T), chosen among species frequently associated with the production of fermented foods. Cultures of these strains were used to inoculate chicory homogenates at about 10^5 CFU/g, which was considered a sufficiently large inoculum to outcompete the autochthonous microbiota of chicory (Bovo et al. 2011), which was determined to be 4.0×10^4 CFU/g.

Microbial growth

Inoculated chicory homogenates were incubated for 48 h to allow a full development of the bacterial population and therefore maximize the production and possible release of compounds of interest. Microbial growth in the chicory cultures was measured by plate counting after 48 h of incubation (Table 2). All strains tested were able to grow on the chicory homogenates, albeit at different levels. All four *Lactobacillus* species grew to $>10^9$ CFU/ml while *St. thermophilus* and *S. cerevisiae* growth just slightly exceeded 10^7 CFU/ml, a value lower than that of the non-inoculated sample, which reached 1.2×10^8 CFU/g. One explanation for this approximately 1-log decrease is that it is the result of an unknown negative interaction or competition between the inoculated strains and the autochthonous microbiota that negatively affected the overall microbial growth.

pH

The starting pH of the chicory homogenate was 5.7. All lactobacilli were able to lower the pH by more than 2 pH units after 48 h of incubation, to values ranging from 3.3 to 3.5 (Table 2). This low value creates a selective environment that could explain the high population numbers reached by all of the strains of this species with respect to the autochthonous microbiota. The pH of the matrix inoculated with *St. thermophilus* did not go below pH 4.5, which is in accordance with its known lower acidifying capability with respect to that of lactobacilli, while *S. cerevisiae*, which does not perform lactic acid fermentation, did not lower the pH significantly, although it is known its potentiality to produce variable amounts of organic acids under specific conditions (Martìnez-Muñoz and Kane 2008). The autochthonous microbiota in the non-inoculated substrate brought the pH down to 4.6, indicating the presence of acidifying microbes, probably LAB, which are normally present in good amounts on plant material.

Scavenging capacity and antimicrobial activity

The radical scavenging activity of the strains was assessed (Tables 2, 3), with *L. hilgardii* and *L. plantarum* giving the best results, well above the other three strains which showed activities just slightly higher than that of the non-inoculated control. As recently reported by Hur et al. (2014), the total antioxidative activity of a fermented plant extract is based on the synergism between polyphenolic compounds and/or other factors, such as microorganisms. A possible explanation is that these LAB have their own antioxidative activities to protect themselves against oxidative damage. As a result, fermenting bacteria might increase the synthesis of the major non-enzymatic antioxidants and free radical scavengers such as glutathione or catalase, as suggested by Spyropoulos et al. (2011).

Antimicrobial activity of fermented plant extract was tested against six indicator strains, namely *B. amiloliquefaciens*, *B. subtilis*, *E. coli*, *Listeria innocua*, *P. fluorescens* and *Staph. xylosus*, chosen among species/strains closely related to pathogenic or spoilage bacteria. Well diffusion tests with supernatants of fermented cultures did not produce visible haloes on the disk plates against any of the indicators tested, revealing the absence of antimicrobial activity towards the chosen indicators, at least at the concentrations applied.

Fermentation of pasteurized chicory homogenates

In order to reduce possible interferences on growth and activity of the introduced strains, all fermentation tests were also performed on pasteurized chicory homogenates (Table 2).

Microbial growth

As expected, the heat treatment heavily affected the resident microbiota (Table 2). Consequently, after 48 h in the non-inoculated sample, the population, probably composed of thermoduric species, reached levels about 50-fold lower than that of the non-pasteurized samples. This decrease indeed did

Table 2 Growth, pH, scavenging activity of the DPPH radical and glucose content of cultures developed in chicory homogenates after 48 h of culture

Strain	CFU/g	pH	Scavenging activity (%)	Glucose concentration (g/l)
Non-pasteurized chicory				
Raw red chicory homogenate[a]	$4.0 \times 10^4 \pm 8.0 \times 10^3$	5.7	10.3 a	ND
Non-inoculated control	$1.2 \times 10^8 \pm 1.1 \times 10^7$	4.6	14.5 a,b	1.33 ± 0.037
S. cerevisiae DSM 70449^T	$1.0 \times 10^7 \pm 3.3 \times 10^6$	5.1	17.6 b	
St. thermophilus DSM 20617^T	$2.2 \times 10^7 \pm 4.0 \times 10^6$	4.5	18.1 b	
L. delbrueckii DSM 20081^T	$3.0 \times 10^9 \pm 6.4 \times 10^8$	3.5	18.1 b	
L. hilgardii DSM 20176^T	$4.0 \times 10^9 \pm 5.5 \times 10^8$	3.3	37.8 c	2.73 ± 0.037
L. plantarum DSM 20174^T	$4.0 \times 10^9 \pm 7.1 \times 10^8$	3.3	36.8 c	2.55 ± 0.005
Pasteurized chicory				
Raw red chicory homogenate[a]	$1.4 \times 10^3 \pm 8.0 \times 10^2$	5.2	17.6 a	ND
Non-inoculated control	$1.0 \times 10^6 \pm 5.5 \times 10^5$	5.0	19.6 a	ND
S. cerevisiae DSM 70449^T	$8.5 \times 10^7 \pm 1.6 \times 10^6$	4.2	12.4 a,b	
St. thermophilus DSM 20081^T	$2.0 \times 10^7 \pm 4.9 \times 10^6$	4.5	15.7 a	
L. delbrueckii DSM 20081^T	$2.9 \times 10^9 \pm 8.5 \times 10^8$	3.6	17.3 a	
L. hilgardii DSM 20176^T	$4.1 \times 10^9 \pm 2.6 \times 10^8$	3.2	37.6 c	2.60 ± 0.04
L. plantarum DSM 20174^T	$4.3 \times 10^9 \pm 1.9 \times 10^8$	3.3	32.8 c	3.22 ± 0.022

Data are reported as the mean of triplicate readings/measurements ± standard deviation (SD) or as the mean without SD. Values followed by the same lowercase letter do not differ at $p < 0.05$

DPPH, 2,2-Diphenyl-1-picrylhydrazyl; ND, none detected

[a] Measured at the beginning of the test

not appear to have an evident impact on the growth of the inoculated strains that reached levels comparable to those of the untreated samples, with the sole exception of *S. cerevisiae* that increased its level by about 1 log.

pH

The heat treatment influenced the initial pH of the homogenate, with an initial decrease to 5.2, followed by a decrease to 5.0 and stabilization after 48 h (Table 2b). In comparison, the final pH of the samples inoculated with bacteria reflected the values of the corresponding non-pasteurized cultures. This is not surprising, since in both cases population levels were similarly high. The growth of *S. cerevisiae* determined a decrease in pH of nearly 1 pH unit with respect to the untreated chicory homogenate. At conditions of very low pH, microbial competition generally favors yeasts, which are more tolerant to acidic pH, leading quickly to the domination of yeasts in the culture (Ribéreau-Gayon et al. 2006). In our study, the resident microbiota seems to have negatively affected the inoculated yeast much more than bacteria, probably due to the relatively high pH.

Antiradical and antimicrobial activities

Radical scavenging activity of all fermented pasteurized substrates was determined (Table 2). Our results do not substantially differ from those of non-pasteurized samples as *L. hilgardii* and *L. plantarum* still proved to be the best performing species, with about twice the level of radical scavenging activity as the other strains tested. Therefore, the previously proposed hypothesis of a synergism between polyphenolic compounds and microorganism species seems also to fit our data.

In terms of antimicrobial activity, the supernatants of fermented cultures did not produce haloes on the culture plates, thus demonstrating the absence of antimicrobial activity, at least at the concentrations used.

Fermentation with autochthonous isolates of *L. hilgardii* and *L. plantarum*

Since *L. hilgardii* and *L. plantarum* showed the strongest scavenging capabilities of the LAB tested, we investigated these species in more detail. *L. hilgardii* is largely found in wines, and many strains are known to exhibit a high alcohol tolerance and a capability to withstand low pH and harsh conditions (Rodas et al. 2005). Its capability to grow well in the presence of polyphenols has also been reported (Alberto et al. 2001). *Lactobacillus plantarum* strains are widespread on plant material. In food processing systems they are frequently used for technological transformations, including the malolactic fermentation of wines (Miller et al. 2011), and are exploited as starter cultures for the fermentation of vegetables and fruits, such as olives, cabbages, carrots and beets (Gardner

et al. 2001; Rodríguez et al. 2009). They are also recovered from meat and dairy products (Vuyst and Vandamme 1994).

Ten *L. hilgardii* (9 autochthonous isolates and the type strain) and 11 *L. plantarum* (10 autochthonous isolates and the type strain) strains were used in our study for the fermentation of pasteurized chicory homogenates (Table 3).

Growth and pH evolution

The growth of the *L. hilgardii* strains in pasteurized chicory is reported in Table 3. The pH was measured throughout a period of 48 h. Six strains lowered the pH to values ranging from 3.7 to 3.9, and three strains reached values of between 4.1 and 4.2. The pH of the pasteurized chicory homogenate cultured with strain TGMMRS06 reached 3.9 just after 24 h, indicating that this strain was the fastest acidifying strain among those tested. The type strain grew significantly better than all natural isolates since it lowered the pH to 3.5 just after 3 h; this efficiency may be related to its origin, since it was isolated from wine, which has pH values of less than 3.5.

The pH of *L. plantarum* cultures was measured throughout a period of 48 h (Table 3). All isolates brought the pH to values well below those of *L. hilgardii*, ranging from 3.2 to 3.5. Moreover, all strains were able to lower the pH to ≤4.0 just after 4 h of incubation. The behavior of the type strain was similar to that of the natural isolates.

These results clearly demonstrate that *L. plantarum* strains have on average a considerable stronger and faster acidifying capability than *L. hilgardii*. The pH of the non-inoculated pasteurized control chicory remained at pH 5.2 throughout the incubation period.

Antimicrobial activity

No antimicrobial activity was observed for all natural isolates of *L. hilgardii* and *L. plantarum* using culture supernatants for the well diffusion assays. Consequently, we concentrated the samples by freeze-drying in order to check possible antimicrobial activity of molecules present at very low concentrations.

Table 3 pH evolution and scavenging activity of the DPPH radical by *L. hilgardii* and *L. plantarum* in pasteurized chicory samples

Sample	pH value after:				Scavenging activity (%) at 48 h[a]
	0 h	4 h	24 h	48 h	
Control	5.5	5.2	5.2	5.2	8.15 ± 0.39
L. hilgardii					
DSM 20176		3.5	3.3	3.3	37.8 ± 1.89
TGMMRS06		5.3	3.9	3.8	8.77 ± 0.44
TGMMRS08		5.3	4.5	3.8	2.61 ± 0.13
TGMMRS11		5.4	4.5	3.8	ND
TGMMRS12		5.2	4.7	3.8	ND
TGMMRS13		5.3	4.8	4.1	6.22 ± 0.31
TGMMRS25		5.3	4.8	4.2	18.12 ± 0.91
TGMMRS27		5.1	4.5	3.9	ND
TGMMRS35		5.4	4.4	3.7	7.43 ± 0.37
TGMMRS41		5.3	4.6	4.1	ND
L. plantarum					
DSM 20174		3.6	3.2	3.2	36.8 ± 1.84
T30PCP01		3.8	3.4	3.2	24.34 ± 1.22
T30PCP05		4.1	4.1	3.4	24.76 ± 1.24
T30PCP06		4.1	4.1	3.3	21.89 ± 1.10
T30PCP07		3.9	3.9	3.3	25.80 ± 1.29
T30PCP14		4.1	4.2	3.2	10.11 ± 0.51
T4MPCP69		3.8	3.8	3.5	8.12 ± 0.41
T4MPCP71		4.0	4.0	3.4	25.95 ± 1.30
T4MPCP73		3.9	4.0	3.4	24.45 ± 1.22
T4MPCP77		4.2	4.1	3.4	24.46 ± 1.23
T4MPCP78		4.0	4.0	3.4	22.48 ± 1.12

ND: none detected

[a] Scavenging activity is presented as the mean ± SD of triplicate measurements

Aliquots of freeze-dried powder (10 mg) of all *L. hilgardii* and *L. plantarum* fermented substrates were tested against six indicator strains, namely *B. amiloliquefaciens*, *B. subtilis*, *E. coli*, *Listeria innocua*, *P. fluorescens* and *St. xylosus* (Table 4).

The control culture did not show any antimicrobial activity against any of the indicator strains, while the inoculated substrates gave differentiated responses.

Supernatant of the type strains of both species tested showed the strongest antimicrobial activity against all of the indicators used.

Some *L. plantarum* and *L. hilgardii* strains did not inhibit any of the indicators, while others were effective against one or more. In particular, *L. hilgardii* TGMMRS08 and TGMMRS12 inhibited five of the six indicators and *L. plantarum* T30PCP01 inhibited all of the indicators tested.

The *L. hilgardii* strains shared some common behaviors, since all inhibited *P. fluorescens* and all but one inhibited *St. xylosus*. Overall, the *L. hilgardii* natural isolates gave almost twice the number of positive results compared with *L. plantarum* (26 vs. 14).

Freeze-dried supernatants (5 mg) of wild strains grown in MRS medium showed antimicrobial activity which disappeared when treated with proteinase K, thus indicating the proteinaceous nature of the inhibitory substance. Interestingly, contrary to all other strains, DSM 20174 supernatant from the MRS culture did not produce any inhibitory activity on the plate disk test, indicating that the antimicrobial activity shown by the DSM 20174 extract is determined by the fermented chicory matrix. To better investigate the strength of the inhibitory activity, we incubated the most susceptible strains, namely *Listeria innocua*, *P. fluorescens* and *St. xylosus*, in liquid cultures in the presence of 2 and 4 mg of extract from chicory fermented by *L. plantarum* DSM 20174. The growth curves, determined over a period of 24 h of incubation at 30 °C, are presented in Fig. 1. In all cases, the growth dynamics of all strains (measured as the increase of turbidity of the cultures) was negatively affected by the presence of the extracts, even at the lower concentrations, although at different rates. When 4 mg was used, the growth appeared to be highly impaired. Although the antimicrobial activity was not evident in the absence of concentration of the extract, chicory

Table 4 Antimicrobial activity of freeze-dried culture supernatants based on the plate disk test results

Sample	*Pseudomonas fluorescens*	*Escherichia coli*	*B. amyloliquefaciens*	*Bacillus subtilis*	*Staphylococcus xylosus*	*Listeria innocua*
Control	–	–	–	–	–	–
L. hilgardii						
DSM 20176	+++	++	+++	+	++	++
TGMMRS06	+	–	–	–	+	–
TGMMRS08	+	+	+	–	+	+
TGMMRS11	+	–	–	+	+	–
TGMMRS12	+	+	+	+	+	–
TGMMRS13	+	–	–	–	+	–
TGMMRS25	+	–	–	–	+	–
TGMMRS27	+	–	–	–	–	–
TGMMRS35	+	–	–	+	+	–
TGMMRS41	+	–	–	+	+	–
L. plantarum						
DSM 20174	+++	++	+++	++	++	++
T30PCP01	+	+	+	+	+	+
T30PCP05	–	–	–	–	–	–
T30PCP06	–	+	–	–	+	+
T30PCP07	–	–	–	–	+	–
T30PCP14	–	–	–	–	–	–
T4MPCP69	–	–	–	–	–	–
T4MPCP71	–	–	–	–	–	–
T4MPCP73	–	+	–	+	–	+
T4MPCP77	–	–	–	–	–	–
T4MPCP78	–	–	–	–	+	–

+++, Inhibition halo > 20 mm; ++, inhibition halo < 20 mm; +, inhibition halo < 10 mm

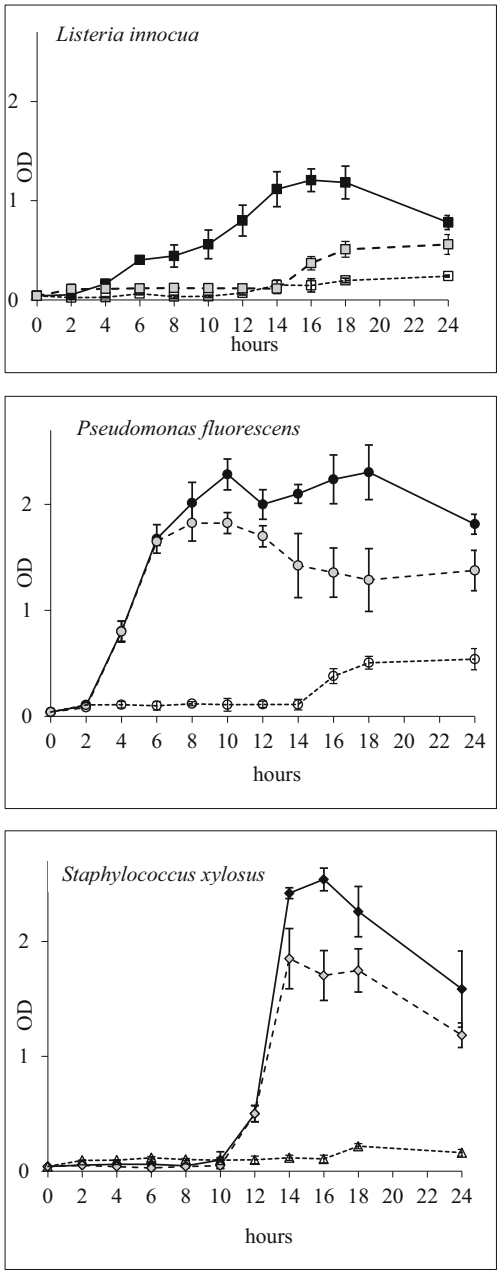

Fig. 1 Effects of the addition of chicory extracts on the growth kinetics of indicator strains. *Continuous lines* No chicory extract, *dashed lines* 2 mg/ml chicory extract, *dotted lines* 4 mg/ml chicory extract

fermentation would appear to be able to reduce pathogen growth.

Antioxidant activity

The DPPH scavenging activity of the samples was calculated (Tables 2, 3) using 5 mg of freeze-dried samples. The fermented samples showed variable behaviors: in those inoculated with *L. plantarum* two strains showed activity nearly as low as that of the control, while the other samples showed quenching activities which were at least threefold higher (22–26 %). The type strain DSM 20174 gave the highest values, close to a fivefold increase in scavenging activity, which was slightly higher than that present at the beginning of the experiment.

With respect to *L. hilgardii* isolates, their quenching activity was revealed to be much lower than those of the *L. plantarum* strains. In particular, three strains did not show any detectable activity, while the remaining showed very low levels, comparable with those of the non-inoculated control, with the sole exception of strain TGMMRS25 that reached 18.12 %. On the contrary, the type strain gave a high value, similar to that produced by the type strain of *L. plantarum.*

In brief, our results indicate that the total antioxidative activity of a fermented plant-based product cannot be predicted on the basis of its total phenolic content alone—rather the synergism between polyphenolic compounds and microbial strains has to be taken into account, as suggested by Naczk and Shahidi (2006).

Phenolic acids profile

Preliminary data on total phenolic content of the non-inoculated pasteurized control samples after 48 h of fermentation showed 132 ± 8 GAE/mg of freeze-dried sample, whereas for the *L. plantarum* and *L. hilgardii* type strain samples, the average phenolic content was 476 ± 43 and 492 ± 51 GAE/mg, respectively, evidencing a higher presence of compounds in the fermented homogenates. Our data are in agreement with those of Hur et al. (2014), thereby confirming the capability of these strains to increase the release of flavonoids during growth.

To evaluate the effect of the strain on the release of polyphenols, we determined the HPLC profile of the phenolic acids in all fermented samples and in the non-inoculated control after 48 h. HPLC analysis of all inoculated samples produced a similar chromatographic pattern. A typical example of the chromatogram of a sample fermented with the *L. plantarum* DSM 20174 type strain is reported in Fig. 2.

It is interesting to note the presence of peak 1, identified as gallic acid; this peak was present in the chicory sample fermented with the *L. plantarum* DSM 20174 type strain but was not detected in the non-inoculated control, which contained only protocatechic acid (peak 2). This finding could be related to microbial tannase activity which catalyzes the hydrolysis of ester bonds into hydrolyzable tannins, releasing gallic acid, as suggested by Rodríguez et al. (2008) and Rodríguez et al. (2009) for strains of *L. plantarum*. Peaks 3 and peak 4 were identified as chicoric and chlorogenic acid, respectively, confirming that chicories contain good amounts of these compounds, as also previously reported by Rossetto et al. (2005) and Heimler et al. (2009). The numerous small peaks appearing between peaks 2 and 3 could be the product of degradation of phenolic compounds, since it is known that

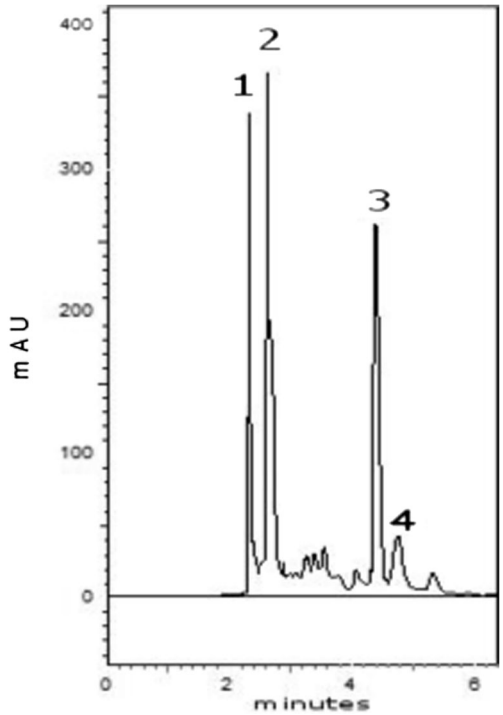

Fig. 2 Representative high-performance liquid chromatography chromatogram of chicory samples fermented with *Lactobacillus plantarum* DSM 20174. *Peaks*: *1* Gallic acid, *2* protocatechic acid, *3* chicoric acid, *4* chlorogenic acid

strains of LAB possess enzymes capable of breaking down higher molecular weight phenolic compounds (Filannino et al. 2015). Consequently,, we conducted a preliminary characterization of these smaller peaks by testing the fractions collected from the extract and searching for a correspondence between phenolic profiles and antioxidant capacity. The extracts were solubilized in distilled water, and the antiradical activity of the fractions was determined. The ability to scavenge the DPPH free radicals was present in those fractions containing peaks 1, 2, 3 and 4, but no activity was detected in the pooled fraction containing the small peaks between peaks 2 and 3.

It has been reported that some strains of *L. plantarum* possess tannase and decarboxylases which enable the degradation of phenolic compounds found in foods (Rodríguez et al. 2008, 2009; Jiménez et al. 2014). The presence of tannase in strains *L. plantarum* and *L. hilgardii* could be advantageous to these microbes as they are found abundantly on plant material, which is known to possess relevant amounts of polyphenols. It is feasible that these bacterial strains have learned to withstand their toxicity and to transform them into nutrient sources. The final products of tannase activity are gallic acid and glucose derived from the glycosylated compounds. To better determine the possible microbial involvement on gallic acid production, we measured glucose content in non-inoculated samples at the beginning of fermentation and after 48 h of fermentation and in samples fermented with *L. plantarum* and *L. hilgardii* type strains (Table 2). Samples with *L. plantarum* and *L. hilgardii* showed considerably higher residual glucose, which could be released from the microbial degradation of glucose-containing polyphenols. In addition, the conversion of glycosides into their aglycone forms by fermentation could be a means of increasing antioxidative activity, as suggested by Tsangalis et al. (2002).

According to Hur et al. (2014) fermentation could represent a good strategy for production or extraction of antioxidant active compounds from natural sources, and new bioactive compounds could be generated during fermentation.

Conclusions

We reported that several microbial species can grow on chicory, with *L. plantarum* and *L. hilgardii* giving the best performances among those tested, and also can provide the fermented substrate with antioxidant and antimicrobial activities. After 48 h of microbial growth, plant-fermented products showed increased antioxidant activity due to the release of phenolic compounds. Moreover, the observed antimicrobial activity was also a result of bacterial growth, since it was found only in fermented samples.

In conclusion, the identification and exploitation of suitable microbial strains for the fermentation process could be used to design functional food from plants historically considered as folk medicine. The potential health benefits of such functional food are open to investigation and would be easier to demonstrate if epidemiological data on the absence of toxicity were available. To our knowledge, our study is the first to examine microbiological exploitation of red chicory by-products with the aim to obtain a fermented product with added beneficial attributes.

Acknowledgments The authors wish to thank F. Fontana and S. Zannoni for their skillful technical assistance.

Ethics statement

Financial statement This study was funded in part by POR "Competitività regionale e occupazione" - parte FESR 2007/2013 Azione 1.1.1. Progetto "RISIB" SMUPR n. 4145 "Potenziamento della rete di infrastrutture a supporto dell'innovazione biotecnologica" and by MIUR (ex-60 % grant).

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

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