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Improved antioxidant capacity of three *Brassica* vegetables by two-step controlled fermentation using isolated autochthone strains of the genus *Leuconostoc* spp. and *Lactiplantibacillus* spp

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ABSTRACT

The aim of this study was to determine the evolution of total phenolic content and antioxidant activity during controlled fermentation of three different Brassicaceae and compare it with spontaneous fermentations. The two-step controlled fermentation was carried out with lactic acid bacteria selected by their biotechnological properties. The selected bacteria were genotypically identified as *Leuconostoc mesenteroides* ssp. *jonggajibkimchii*, *Ln. mesenteroides* ssp. *dextranicum*, *Lactiplantibacillus plantarum* ssp. *argentoratensis*, *L. plantarum* and *L. pentosus*. The total phenolic content did not show a trend when comparing the different fermentations; it depended on the type of extract and vegetable. The controlled fermentation exhibited higher antioxidant activity than spontaneous fermentations for all the vegetables during the process. The extracts of red cabbage exhibited a total phenolic content and antioxidant activity higher than chinese and white cabbage, regardless of the type of fermentation.

1. Introduction

The research on the potential health benefits of plant polyphenols as biological antioxidants has increased in recent years. Biological antioxidants are considered molecules that, when present in small concentrations, preserve, reduce or prevent the oxidative stress of the biomolecules (Hur et al., 2014).

Phenolic compounds of plants are secondary metabolites that can act as reducing agents (free radical chain terminators), singlet oxygen quenchers, metal chelators, and hydrogen donors (Apak et al., 2016). Furthermore, epidemiological studies strongly show that prolonged diets rich in polyphenols provide significant protection against the development of degenerative diseases related to oxidative stress (Pandey & Rizvi, 2009).

Among vegetables, Brassicaceae species are frequently recommended for a healthy diet because they are rich in fiber, minerals, and bioactive compounds such as polyphenols and glucosinolates. Recent studies propose that diets rich in different cabbage species are related to a lower risk of some types of cancer, allowing the recognition of these vegetables as functional foods (Šamec & Salopek-Sondi, 2018).

Vegetable spontaneous fermentation is a method of traditional and cultural preservation; it takes place by the lactic acid bacteria (LAB) present in the vegetable tissue (Di Cagno et al., 2013), which convert carbohydrates mainly into organic acids. Lactic acid fermentation of vegetables not only improves sensory properties and the safety of the foods but also reduces the anti-nutritional factors and enhances the health-promoting synthesis of bioactive compounds (Di Cagno et al., 2013).

Bacterial cell wall degrading enzymes play an essential role by facilitating the release of bioactive compounds during vegetable fermentation. Furthermore, the increment of the antioxidative activity is also associated with the structural changes in phytochemicals that take place in the process. Enzymatic activity and lactic acid produced by LAB during fermentation contribute to the increase of the depolymerization and conversion of high molecular weight phenolic compounds producing metabolites with higher reducing activity (Hur et al., 2014).

Spontaneous fermentation of vegetables relies on a sequential process that includes hetero and homo-fermentative LAB, complemented or

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not with yeasts. The main LAB species isolates are *Leuconostoc*, *Lactiplantibacillus*, *Weissella*, *Enterococcus*, and *Pediococcus* genera. The epiphytic microbiota on vegetables is variable and depends on different factors; hence spontaneous fermentation may not guarantee the same performance during processing. Inadequate inhibition of pathogens, microbial spoilage, and undesirable changes in the nutritional, rheology, and sensory properties are some of the risks of spontaneous fermentation (Di Cagno et al., 2013).

Controlled fermentation is the alternative method to overcome the instability in vegetable fermentation and product variability. Recently, the use of starter cultures has been implemented in vegetable fermentation with two main alternatives: the use of allochthonous or autochthonous starters. Although there are several commercial/allochthonous starters, these may present limitations because they were not previously selected to ferment a specific vegetable, where the intrinsic and extrinsic parameters change. Di Cagno et al. (2013) have recommended the use of autochthonous starters for vegetable fermentation since they ensure targeted sensory, nutritional, and rheology properties and prolonged shelf life.

To our knowledge, there are no reports of the whole process from the isolation and selection of LAB until their application as starters in vegetable fermentations. Given this panorama, the goal of this study was the isolation of LAB strains during different steps of spontaneous fermentation of *Brassica* vegetables and further selection based on their technological properties. The selected strains were used in a two steps-controlled fermentation of white cabbage, red cabbage, and chinese cabbage. The evolution of antioxidants activity and the total phenolic content (TPC) were compared with those obtained in the spontaneous fermentation.

2. Materials and methods

2.1. Isolation of LAB from spontaneous cabbage fermentation

The strains were isolated periodically during spontaneous fermentation of three Brassica vegetables: white cabbage (*Brassica oleracea* var. *capitata* f. *alba*), chinese cabbage (*B. rapa* var. *glabra* Regel), and red cabbage (*B. oleracea* var. *capitata* f. *rubra*). The vegetables were bought from a local farm of Valle Inferior del Río Chubut located in Patagonia, Argentina (-43.14 latitude, −65.19 longitude, elevation 11 m above sea level). The cabbages were planted in February 2021 and harvested in June 2021. Before the fermentation, the dry outer leaves of the bulbs were eliminated, and the cabbages were cleaned and chopped into 2 mm thick strips. Then, 1.7 Kg of each processed vegetable was mixed with 3.0 % (w/w) common salt and placed in airtight glass jars (2 L). The fermentation process was carried out at 18 °C for 30 days for each vegetable following the recommendations of Thakur et al. (2017).

Isolation was carried out by serial dilution using the pour plate method. Solid samples were collected the days 0, 1, 2, 3, 4, 5, 10, 15, 20, 25, and 30 during the spontaneous fermentations. The solid sample was resuspended in sterile saline water (0.85 %) at a ratio of 1:1 w/v and homogenized in a vortex for 1 min. Bacterial populations were determined by surface plating on Man, Rogosa, and Sharpe (MRS) agar (Biokar, Beauvais, France), and MRS agar supplemented with vancomycin (Fada Pharma S. A., Buenos Aires, Argentina) (15 μg/mL). The plates were incubated at 28 °C for 48 h. Colonies that displayed different morphologies were picked up and purified. All isolates were initially examined for Gram reaction and production of catalase and oxidase. Isolates were conserved at −30 °C in tryptic soy broth (Britania, Buenos Aires, Argentina) medium supplemented with 10 % (v/v) of glycerol (Anedra, Buenos Aires, Argentina) as a cryoprotectant.

2.2. Technological properties

2.2.1. Salt and pH acid tolerance

All the isolates were subjected to stress conditions to obtain qualitative data about their salt and acidity tolerance. An aliquot of the overnight cultures was added into MRS broth (1:10 dilution), supplemented with 3 % NaCl (Anedra, Buenos Aires, Argentina) and adjusted to pH 3.5 with lactic acid (Sigma Aldrich, St. Louis, USA). Then it was incubated at 30 °C for 48 h. Afterward, a 5 μL aliquot of the culture was plated on MRS agar plates and incubated at 30 °C for 72 h; the development of colonies was interpreted as a positive resulted.

2.2.2. Influence of phenolics on the growth of lactic acid bacteria

To study the effects of phenol, tannic acid, and gallic acid on the growth of LAB, the method proposed by Ajaz et al. (2004) was carried out with some differences. Overnight cultures (5 μL) were used to inoculate MRS agar plates containing 5 g/L phenol (Biopack, Buenos Aires, Argentina), 1 g/L tannic acid (Biopack, Buenos Aires, Argentina), and 3 g/L gallic acid (Biopack, Buenos Aires, Argentina), individually. The plates were incubated at 30 °C for 72 h. The growth of colonies at 72 h was considered a positive result.

2.2.3. Exopolysaccharide production

The strains were cultured on Congo red agar (Biopack, Buenos Aires, Argentina) using a method modified by Freeman et al. (1989). The plates were incubated at 30 °C for 24 h. Exopolysaccharide production was detected by the formation of black colonies in the medium.

2.2.4. Tannase and pectinase activity

The ability of the strains to degrade tannic acid was evaluated by a qualitative method. Briefly, the isolates were streaked on MRS agar plate formulated without carbohydrates and supplemented with tannic acid (1 g/mL) and purple bromocresol 0.005 % (w/v) (Sigma Aldrich, St. Louis, USA) as pH indicator. The plates were incubated at 30 °C for 48 h. After incubation, the formation of a yellow zone around the colonies was regarded as positive tannase activity.

Then, the screening of extracellular pectinase activity was evaluated by plate assay as previously described by Karthik et al. (2011). The isolates were sowed in the yeast extract pectin medium containing pectin 2.5 g/L (Sigma Aldrich, St. Louis, USA), yeast extract 5.0 g/L (Britania, Buenos Aires, Argentina), supplemented with 2 % agar (Britania, Buenos Aires, Argentina). Plates were incubated at 30 °C for 48 h. Then, the results were revealed following the instructions of Vidhyasagar et al. (2013). Plates were stained with 0.1 % (w/v) aqueous Congo red for 10 min and then washed with 1 M NaCl solution. The clearance of the zone around the colony after staining was considered as positive enzymatic activity.

2.3. Genotypic identification

Molecular identification of selected LAB isolates was conducted by amplification of the 16S rRNA gene. In the first stage, total genomic DNA was extracted using Wizard Genomics kits (Promega, Madison, Wisconsin, EE.UU) following the manufacturer's instructions. Afterward, to amplify the 16S rRNA bacterial gene, universal primers 27F (5′-AGAGTTTGATCCTGGCTCAG-3′) and 1492R (5′-GGTTACCTTGTTACGACTT-3′) were used (Lane, 1991). Both strands of the PCR products were sequenced using the commercial services of Macrogen Inc. (Seoul, Korea). The homologous sequence searching was carried out using the BLAST algorithm (https://www.ncbi.nlm.nih.gov/BLAST/).

2.4. Lactic starter cultures

Leuconostoc mesenteroides ssp. *jonggajibkimchii* RCTw1.1, *Ln. mesenteroides* ssp. *dextranicum* RBTw100, *Lactiplantibacillus plantarum* ssp. *argentoratensis* RBTw102, *L. plantarum* AKTw180; *L. pentosus* AKTw332 were selected for the controlled fermentation trial. The organisms were subcultured in MRS broth at 30 °C for 18–24 h and used for the controlled fermentation studies.

2.5. Controlled fermentation trial

The controlled fermentation trial was carried out with the same vegetables (chinese cabbage, white cabbage, and red cabbage). These were obtained and processed in the same way as mentioned above. Nevertheless, each salted cabbage jar was heated at 100 °C for 5 min, cooled at room temperature, and inoculated with strains belonging to the genus *Leuconostoc* (3×10^5 CFU/g of each strain) and fermented at 18 °C for three days. The following fermentation step involved adding the second starter with strains belonging to the genus *Lactiplantibacillus* (3×10^5 CFU/g of each strain) and incubating for the next 27 days. The second inoculum was added when the pH was reduced to ~ 4, as suggested by Jagannath et al. (2012).

The spontaneous fermentation of chinese cabbage, white cabbage, and red cabbage were carried out under the same conditions mentioned for the LAB isolation. The fermentations of the vegetables were performed in triplicate.

2.6. pH and lactic acid bacteria count

The pH and LAB count were monitored on days 0, 1, 3, 5, 10, 15, 20, 25, and 30 of the fermentation process. A pH meter (model Orion 410A, Cole-Palmer, St. Neots, UK) was used to measure the pH during the fermentation. The growth of LAB was monitored on MRS agar using serial dilutions of solid sauerkraut samples, and the plates were incubated at 30 °C for 48 h. The results were expressed in CFU/g sample. All trials were performed in triplicate.

2.7. Preparation of solvent extracts

The samples collected on days 0, 1, 3, 5, 10, 15, 20, 25, and 30 were dried on a stove for three days at 37 °C and then ground. Methanol (Biopack, Buenos Aires, Argentina) and distilled water were used as solvents using a solid to liquid ratio of 1:10 w/v. For water extract, the mixtures were autoclaved for 15 min at 105 °C, while methanol ones were incubated at 37 °C for 3 h with agitation. Finally, the extracts were centrifuged (13.000 × *g*) for 15 min, and the supernatants were stored at −20 °C.

2.8. Measurements of the total phenolics

The Folin-Ciocalteau method was used to determine the TPC with minor modifications (Agbor et al., 2014). The assay was carried out by mixing 50 μL of the extract with 100 μL Folin–Ciocalteu﷽s phenol reagent (Sigma Aldrich, St. Louis, USA). After ten minutes, 2 mL Na_2CO_3 (1.0 % w/v) was added and allowed to react for 90 min at 25 °C. The absorbance was measured with a spectrophotometer (Jenway, Cole-Palmer, St. Neots, UK) at 750 nm. A calibration curve was carried out using gallic acid (50–800 μg/mL) as standard. The results were expressed as milligram gallic acid equivalents per 100 g dry weight (mg GAE/100 g DW).

2.9. Measurements of antioxidant activity

2.9.1. DPPH radical scavenging assay

The free radical scavenging activity of extracts was evaluated by DPPH, according to the method previously described by Chen et al. (2005) with some modifications. An aliquot of 100 μL of the sample was mixed with 900 μL of an DPPH ethanolic solution (100 μM) (Sigma Aldrich, St. Louis, USA) and was incubated for 30 min at 25 °C in darkness. Then, the absorbance of the reaction was measured in a spectrophotometer at 517 nm. A standard curve of ascorbic acid (10.5–176 μg/mL) was constructed as a reducing agent. The results were expressed as milligram ascorbic acid (Sigma Aldrich, St. Louis, USA) equivalents per 100 g dry weight (mg AAE/100 g DW).

2.9.2. Cupric reducing antioxidant capacity (CUPRAC) assay

The antioxidant activity of the extracts was determined by the assay CUPRAC, according to the recommendations of Gouda and Amin (2010), with modifications. A reaction mixture was prepared with 1 mL of $CuCl_2$ (0.01 M) (Cicarelli, Córdoba, Argentina), 2 mL of Neocuproine solution (5 mM) (Sigma Aldrich, St. Louis, USA), and 3 mL of acetate buffer (50 mM, pH 5.0) (Sigma Aldrich, St. Louis, USA). Then, 900 μL of the reaction mixture was mixed with a 100 μL sample; it was manually shaken and incubated for 1 h in darkness. The absorbance was measured in a spectrophotometer at 450 nm, and was carried out a calibration curve with ascorbic acid as standard (10.5–176 μg/mL). The results were expressed as mg of ascorbic acid equivalent per 100 g of dry weight (mg AAE/100 g DW).

2.10. Statistical data analysis

All assays were carried out in duplicate, unless otherwise mentioned. The data were subjected to one and two-way analyses of variance (ANOVA) at a significance level of $\alpha = 0.05$. One way ANOVA was performed to evaluate the effect of time or type of vegetable on LAB, pH, TPC, DPPH and CUPRAC. The two-way ANOVA on the response variables TPC, DPPH, and CUPRAC were performed to check the existence of effects of the type of fermentation, the time, and their interaction, partitioning for the type of extract and cabbage. Tukey's procedure determined significant differences between means at $p \leq 0.05$. InfoStat statistical software was used for all data analysis.

3. Result and discussion

3.1. Isolation of LAB from spontaneous cabbage fermentation

One hundred and twenty bacterial strains were isolated from the spontaneous fermentation of *Brassica* vegetables (chinese, white and red cabbage). All bacterial isolates fit LAB classification as Gram-positive, catalase, and oxidase negative.

3.2. Technological properties

Since LAB that compose the microbiota of fruit and vegetables are variable and do not guarantee a similar performance in the process, the selection of strains based on technological properties has become a new tool in the food industry. Nowadays, commercial starter cultures have begun to be used to improve and accelerate the fermentation process of vegetables and fruits (Di Cagno et al., 2013). In this study, the strains selected were expected to display technological properties, including the ability to adapt to the fermentation environment. The NaCl concentration and pH are critical factors in the fermentation process of vegetables. The NaCl induces the plasmolysis in plant cells, incremented anaerobic conditions to the proper development of the LAB. While the pH plays a role in the preservation and the adequate development of sensory characteristics of the fermented product (Swain et al., 2014). Out of 120 LAB isolates, 90 strains showed tolerance to an environment of 3 % NaCl (w/v) and pH 3.5. LAB generally tolerate high salt concentrations and low pH; these properties give them an advantage over other microorganisms during plant fermentation.

Starter cultures must adapt to growth in raw vegetable material where phenolic compounds are found in high concentration (Pandey & Rizvi, 2009). In this report, the effects of phenolic compounds on the growth of the strains selected above (90) were evaluated. The isolates showed different degrees of sensitivity towards these compounds; 80 strains showed tolerance to gallic acid, 75 fronted to tannic acid, while 73 resisted to phenol. Some LAB species such as *L. plantarum*, metabolize phenolic compounds by reduction and/or decarboxylation (Hur et al., 2014). Although, the tolerance to phenol acids by lactobacilli is strain and species specific (Sánchez-Maldonado et al., 2011).

The production of exopolysaccharide by LAB has been applied in the

dairy industry for a long time, while the use in the vegetable fermentation process is most recent. Recent reports indicate that exopolysaccharide production can contribute to improve the rheological properties of the fermented vegetable product. Moreover, these can exert antioxidant, cholesterol-lowering, and anti-inflammatory activity, generating positive health effects on the consumer (Guérin et al., 2020). A total of 74 of 90 strains preselected showed exopolysaccharide production on Congo Red Agar.

Besides, the pectinase and tannase activity of LAB strains using plate assays were evaluated. Out of 90 LAB preselected, 71 isolates displayed pectinase activity, while only 49 strains showed tannase activity. These enzymes contribute to the softening of vegetable tissues and allow the release of nutritional compounds. Moreover, pectinases break down pectins present in plant tissues in galacturonic acid, causing an increase in carbon sources (Chatterjee et al., 2016). The tannase specifically breaks the galloyl ester bonds of tannins, preventing the formation of antinutritional complexes with proteins (Di Cagno et al., 2013; Hur et al., 2014). Besides, the activity of this enzyme positively influences antioxidant activity (Hur et al., 2014).

Among the isolated strains, five were selected on the basis of their technological properties (Table 1): RCTw1.1, RBTw100, RBTw102, AKTw180, AKtw332. It can be observed that all selected *Lactobacillus* strains displayed tolerance to tannic acid, gallic acid, and phenol. On the other hand, all the *Leuconostoc* strains studied in this trial were sensitive to the phenol concentration tested (0.5% v/v). *Leuconostoc* RCTw1.1 and RBTw100 were selected based on their higher acidification rate (data not shown) and the metabolic properties assayed. *Leuconostoc* species are predominant at the first stages of the process, then the population decrease as the fermentation proceeds, and *Lactiplantibacillus* species markedly increase through the following stages. This feature can be attributed to the low resistance of *Leuconostoc* species against phenolic compounds that increased antimicrobial activity at lower pH. Moreover, *Leuconostoc* species cannot reduce phenolic acids; hence it is not possible NAD + regeneration by reoxidation of NADH by this mechanism, which means a less efficient energy supply (Filannino et al., 2014).

3.3. Genotypic identification

Molecular identification of selected LAB strains was conducted by amplification of the 16S rRNA gene and their subsequent sequencing. The sequences 16S rDNA of strains were employed for bacterial identification through alignment with the 16S rDNA sequences from the GenBank database (website). Sequence analysis of 16S rDNA showed that isolate RCTw1.1, RBTw100, AKTw180, and AKTw332 exhibited 100 % sequence similarity with *Leuconostoc mesenteroides* ssp. *jonggajibkimchii* strain DRC1506, *Ln. mesenteroides* ssp. *dextranicum* strain JCM 9700, *Lactiplantibacillus plantarum* strain JCM 1149, *L. pentosus* strain JCM 1558, respectively. While 16S rDNA sequence of the strain RBTw102 exhibited 99 % homology with *L. argentoratensis* strain JE10. The sequences were submitted to GenBank with the access numbers MT702992, MT178435, MT178436, MT178440, and MT178445.

3.4. pH and lactic acid bacteria count

The chinese cabbage, white and red cabbage were fermented individually through spontaneous and controlled fermentation for 30 days at 18 °C. A temperature of 18 °C was selected for carrying out the two fermentations because previous reports recommend a range of 16–20 °C for the optimum growth of fermentative microorganisms; higher temperatures (>20 °C) accelerate acid production and may affect the flavor of the products, while lower temperatures (<15 °C) produce slow and incomplete fermentations (Erkmen & Bozoglu, 2016). Counts of LAB and pH measurement were used to monitor the evolution of the fermentation process.

The two types of fermentation displayed a similar pattern in the different vegetables with some differences (Fig. 1). In spontaneous fermentation, the LAB counts were ≈3.5 log CFU/g with a pH of ≈6 at the start of the process. The values obtained are in agreement with previous reports (Di Cagno et al., 2013). Then, the LAB cell count increased quickly, and after three days, they reached a peak level of ≈7.8 log CFU/g, while pH values dropped to ≈4.5 in the different vegetables. Later, LAB count dropped quickly to ≈6 log CFU/g after ten days of fermentation, and remained stable until the 30 days of process ($p > 0.05$) in chinese and white cabbage, while in red cabbage, the cell count decreased markedly after 20 days with final values of < 5 log CFU/g. Di Cagno et al. (2013) reported that the rapid decrease of the LAB is the result of their sensitivity to the acid conditions. The pH decrease was quite similar for chinese and white cabbage; the lowest values were observed at five days of fermentation (pH≈4.3) and remained stable over 30 days ($p > 0.05$). Red cabbage fermentation did not exhibit a sharp decrease of pH; the lowest value was achieved after 15 days (pH≈3.9) of incubation and remained stable as in the case of chinese and white cabbage.

The epiphytic flora was previously reduced with heat treatment in the controlled fermentation trial. Afterward this treatment the mixture of selected strains of *Leuconostoc* species was added (≈5 log CFU/g). After three days of incubation at 18 °C, when the cell counts increased to > 8.0 log CFU/g, and the pH values decreased to ≈4.0, the inoculum composed by the selected *Lactiplantibacillus* strains was added (≈5 log CFU/g). Afterward, decreases of the LAB counts were observed gradually for the three cabbages, reaching a final value of < 4.8 log CFU/g.

The initial pH displayed comparable values in the two types of fermentation ($p > 0.05$) for each vegetable. While after 24 h of process, pH dropped more quickly in controlled fermentation, achieving constant values at ten days for red and white cabbage, and at 15 days for chinese cabbage ($p < 0.05$). These differences between the processes could be attributed to the selected strains inoculations carried out in the controlled process in agreement with previous works that reported a significant drop in pH when *Ln. mesenteroides* was used as the starter in vegetable fermentations (Gardner et al., 2001). At the end of the spontaneous and controlled process, the pH values were comparable for red and chinese cabbage but not for white cabbage ($p > 0.05$). It must be taken into account that a higher acidification rate is associated with greater food safety and structural modifications in the phytochemical compounds (Hur et al., 2014).

Table 1
Selected strains on the basis of their technological properties.

Assay	RCTw1.1*	RBTw100*	RBTw102*	AKTw180*	AKTw332*
Salt and pH acid tolerance	+	+	+	+	+
Tolerance phenol	–	–	+	+	+
Tolerance tannic acid	+	+	+	+	+
Tolerance gallic acid	+	+	+	+	+
Exopolysaccharide production	+	+	+	+	+
Tannase activity	+	+	+	+	+
Pectinase activity	+	+	+	+	+

*RCTw1.1: Leuconostoc mesenteroides ssp. jonggajibkimchii; RBTw100: Ln. mesenteroides ssp. dextranicum; RBTw102: Lactiplantibacillus argentoratensis; AKTw180: L. plantarum; AKTw332: L. pentosus.

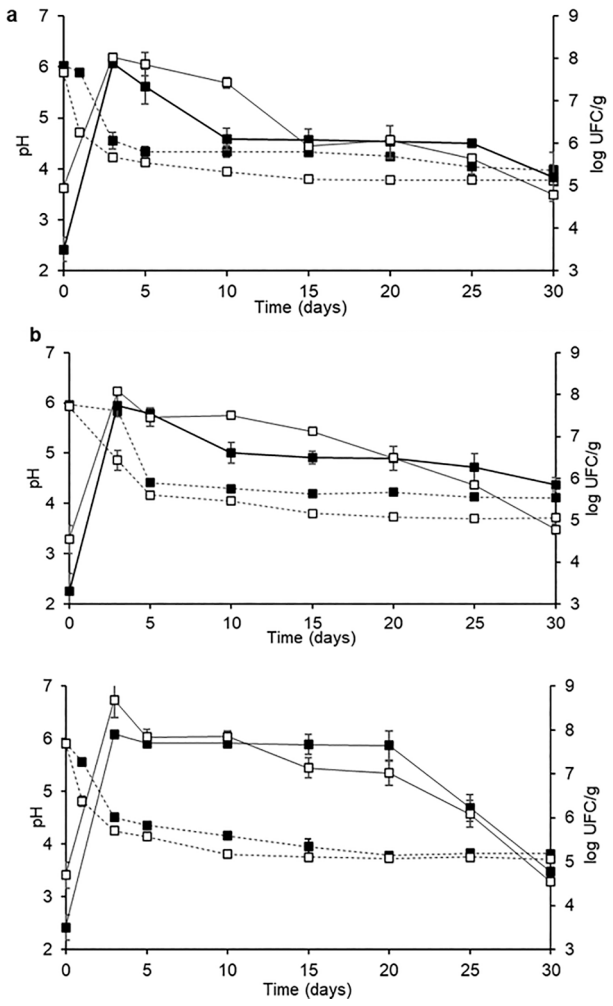

Fig. 1. pH changes (– – –) and total lactic acid bacteria (–) counts grown on MRS agar determined in cabbage during spontaneous (■) and controlled fermentation (□) of chinese cabbage (a), white cabbage (b) and red cabbage (c). Each value is mean ± standard deviation of two measurements.

3.5. Measurements of the total phenolics

The TPC in chinese, white, and red cabbage extracts (methanol and water) during controlled and spontaneous fermentations can be observed in Fig. 2. The water extracts displayed higher values of TPC than the methanol ones, independent of the cabbage species (Fig. 2, left vs right panels) and fermentation type. The phenolic compounds are polar molecules hence their extraction is highly influenced by polarity (Hosseini et al., 2016). Among the solvents used for the assay, water was more efficient than methanol due to higher polarity (dipole momentum of water and methanol are 1.95 and 1.65 D, respectively). Furthermore, red cabbage extracts exhibited a higher TPC than chinese and white cabbage ($p < 0.05$). Previous works report similar values; 1851 mg GAE/100 g DW in red cabbage (Tabart et al., 2018), 980–1220 mg GAE/100 g DW in white cabbage (Vicas et al., 2013), and 347.46 mg GAE/100 g DW in chinese cabbage (Seong et al., 2016).

Chinese cabbage exhibited a similar behavior in both fermentation types, TPC (water and methanol extracts) showed a significant increase until the five days of fermentation; then, the values slightly decreased ($p < 0.05$) (Fig. 2a and 2b). In the controlled fermentation of white cabbage, water extract exhibited a significant increase in TPC on day one and remained stable until the end of the process ($p > 0.05$). While in spontaneous fermentation, no significant variations were observed ($p > 0.05$) (Fig. 2c). In contrast, the methanol extract displayed relatively stable values of TPC during controlled fermentation ($p > 0.05$), while the spontaneous fermentation showed a significant increase in the last days ($p < 0.05$) (Fig. 2d). A similar trend was reported by Zubaidah et al. (2020), which showed an increase of TPC on day 5 in controlled fermentation of white cabbage inoculated with *Ln. mesenteroides* and *L. plantarum* strains. The controlled fermentation of chinese cabbage showed a TPC higher than spontaneous fermentation at the end of the process in both extracts ($p < 0.05$). On the other hand, in white cabbage, no significant differences were observed between the final TPC values in both fermentations.

Regarding red cabbage, methanol extracts displayed a slightly increased TPC during the first day ($p < 0.05$) and then remained relatively stable until the end of the process in spontaneous fermentation. In the case of controlled fermentation TPC values showed an upward trend but it was not significant ($p > 0.05$) (Fig. 2f). As the methanol ones, water extracts increased slightly on the first day of the process ($p > 0.05$); in spontaneous fermentation, the values remain relativity stable until the end. Meanwhile, the TPC ones in the controlled fermentation dropped until day 15 (Fig. 2e). Unlike the other vegetables, the spontaneous fermentation of red cabbage displayed higher values of TPC than controlled fermentation in both extract types at the end of the processes ($p < 0.05$). The difference in TPC due to the type of fermentation could depend on the enzymatic activity of LAB involving each process and the consequent ability to metabolize the phenolic compounds of high-molecular-weight and thus obtain a reduction of TPC (Hunaefi et al., 2013).

The results obtained suggest that the increasing organic acids and enzymatic hydrolysis of LAB during fermentation could be involved in the degradation of phenolic compounds. The extension of the process depends on the type of fermentation and vegetable used. Vegetable phenolic compounds have great structural diversity; however, they are mainly linked to other molecules such as sugars, forming glycosides. During fermentation, the polyphenols can be transformed to aglycone forms by the action of LAB enzymes, producing molecules with higher antioxidant activity. *L. plantarum* strains possess tannase activity able to hydrolyse ester bonds present in hydrolysable tannins, releasing powerful antioxidant compounds such as gallic acid and pyrogallol (Hur et al., 2014). Furthermore, pectinases may change cabbage texture during fermentation (Seong et al., 2016), allowing the release of phenolic compounds.

3.6. Measurements of antioxidant activity

DPPH radical scavenging assay is an electron transfer-based method and is the most frequently used to determine antioxidant activities of phenolic compounds (Apak et al., 2016) (Shahidi & Zhong, 2015). Fig. 3 depicts the antiradical activity of water (left panels) and methanol (right panels) extracts of cabbages studied against DPPH radical, differentiating the type of fermentation. The methanol extracts contained significantly lower reduction power than the water ones in all cabbages in controlled fermentation ($p < 0.05$). While the spontaneous process did not show significant differences ($p > 0.05$) between water and methanol extracts throughout the fermentation period.

Red cabbage displayed the highest antioxidant capacity, reaching 567.53 ± 16.80 mg AAE/100 g DW and 438.51 ± 14.66 mg AAE/100 g DW in water (Fig. 3e) and methanol extracts, respectively (Fig. 3f), after 30 days of controlled fermentation. Meanwhile, chinese cabbage reached maximum values of 199.58 ± 7.76 mg AAE/100 g DW and 147.59 ± 4.35 mg AAE/100 g DW in water and methanol extract, respectively, at the end of the controlled process (Fig. 3a and 3b). White cabbage reached maximum values of 202.87 ± 7.24 mg AAE/100 g DW and 172.60 ± 7.64 mg AAE/100 g DW in water and methanol extract, respectively, for same time of controlled fermentation (Fig. 3c and 3d). Upadhyay et al. (2016) reported that fresh red cabbage exhibited higher antioxidant activity than other *Brassica* vegetables studied as white cabbage; the values obtained in this assay showed that this difference

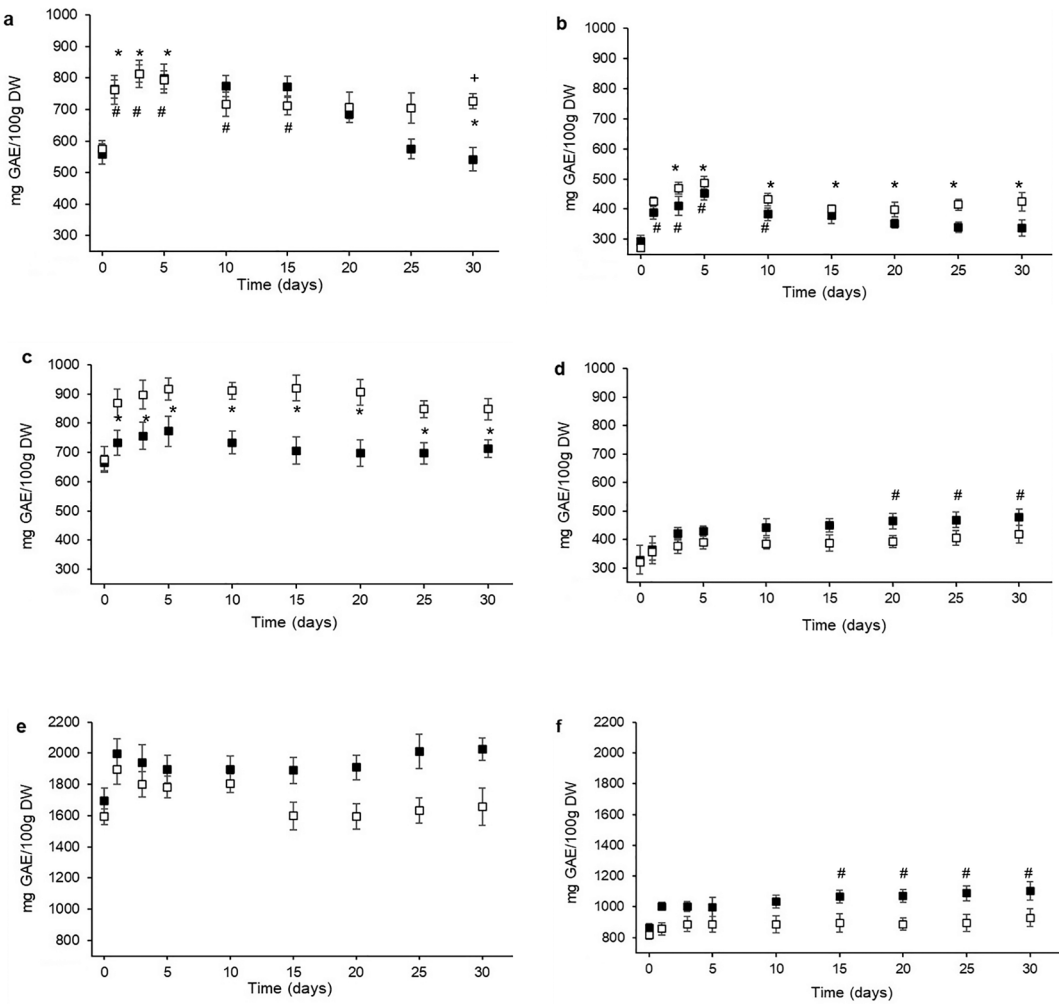

Fig. 2. Total phenolic content in extracts water (a, c, e) and methanol (b, d, f) of chinese cabbage (a, b), white cabbage (c, d) and red cabbage (e, f); during spontaneous (■) and controlled fermentation (□). Each value is mean ± standard deviation of three measurements. *Significant difference from initial time for controlled fermentation; #significant difference from initial time for spontaneous fermentation; +significant difference between the type of fermentation-time (interaction).

between the vegetables continue after the fermentation process.

Regardless of the extract type, the controlled fermentation exhibited highest antioxidant capacity than spontaneous fermentation in chinese and white cabbage ($p < 0.05$). The water extracts of red cabbage also displayed the same behavior, while the methanol extracts did not show significant differences between both fermentations ($p > 0.05$).

In the controlled fermentation, antioxidant activity increased continuously until day 5 and, then remained stable in all the vegetables. Zubaidah et al. (2020) reported a similar trend during the controlled fermentation of white cabbage, highlighting the increase of antioxidant activity on day 5 of the fermentation process for the different combinations of *Ln. mesenteroides* and *L. plantarum* strains assayed.

In contrast, the spontaneous fermentation showed variations according to the type of extract and vegetable. The antioxidant activity of the water extracts of the three cabbages did not show significant increases during the period of fermentation. The antioxidant activity of the methanol extracts exhibited a significant increase after 15 days and then remained stable in white cabbage, while chinese and red cabbage did not show significant differences during the process. Kusznierewicz et al. (2008) reported that during spontaneous fermentation of white cabbage, the antioxidant activity moderately increases and reaches a plateau after about ten days.

CUPRAC is a copper reduction assay developed as a variant of the FRAP method; it is an electron transfer-based method (Apak et al., 2016). The values of AAE obtained with the CUPRAC assay were higher than the obtained with the DPPH method in all cases (Fig. 3 vs Fig. 4). These differences are related to the ability of each method; the CUPRAC assay simultaneously measures hydrophilic and lipophilic antioxidants of the samples, while the DPPH assay only detects molecules soluble in organic solvents as alcohols (Apak et al., 2016).

Regarding white and red cabbage, their water extracts exhibited an antioxidant capacity significantly higher than the one from their methanol extract (Fig. 4c vs 4d, 4e vs 4f) ($p < 0.05$). Whereas chinese cabbage did not show differences between extracts (Fig. 4a vs 4b) ($p > 0.05$). Boğa et al. (2011) used CUPRAC assays to determine the antioxidant activity of the aqueous and ethanol extract of red cabbage, and they found that water extract exhibited a higher antioxidant level than ethanol ones. It is well known that antioxidant activity in vegetable matrices depends on growing conditions, region, weather conditions, harvest time, and storage time (Aires et al., 2011).

The antioxidant capacity of methanol and water extracts of chinese cabbage increased significantly during the first day and then decreased until 10 days; the values remained relatively stable until day 30 in spontaneous fermentation, while it increased slightly at the end of the controlled process (Fig. 4a, b). At the end of the process, the controlled fermentation exhibited a higher activity antioxidant than spontaneous fermentation for both extract types ($p < 0.05$).

The methanol extracts of white cabbage showed an increase in

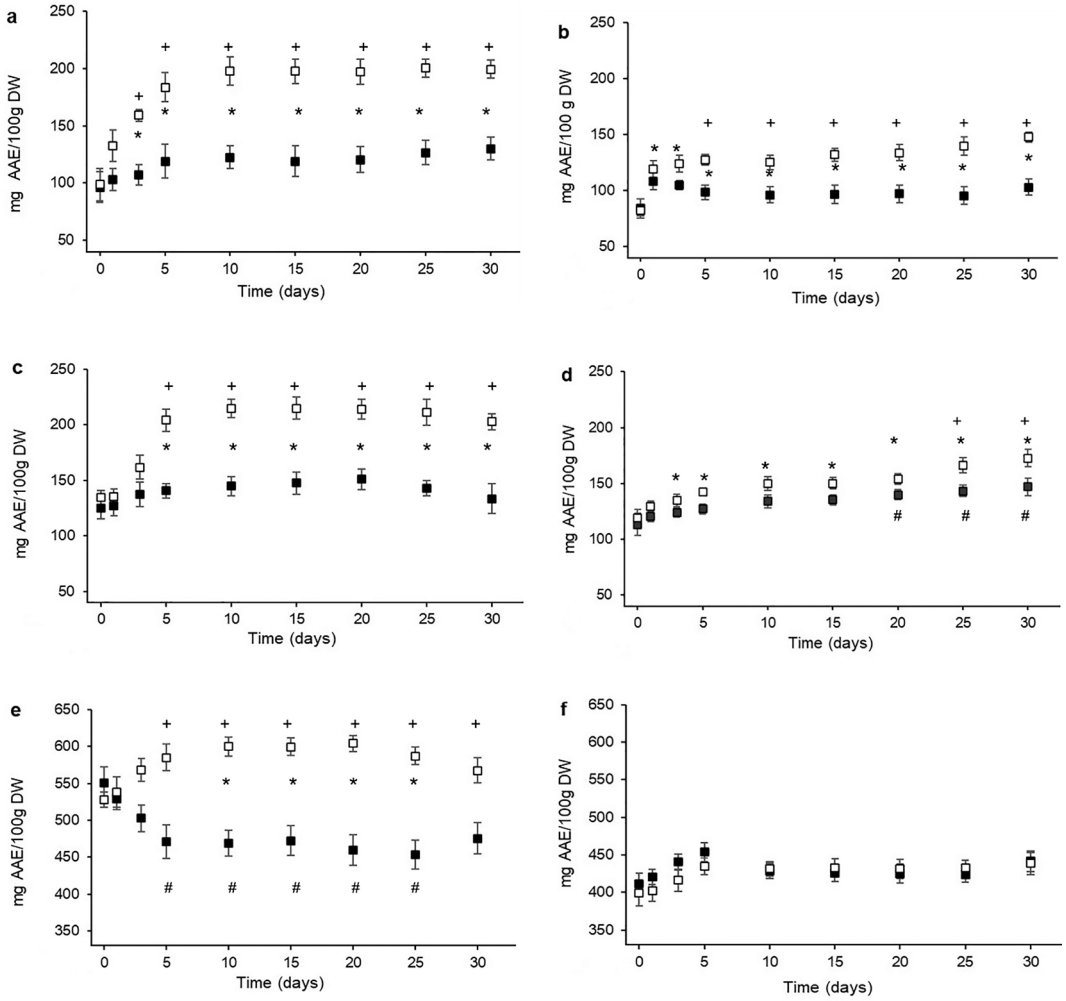

Fig. 3. Antioxidant activity (DPPH assay) in extracts (water (a, c, e) and methanol (b, d, f) of chinese cabbage (a, b), white cabbage (c, d) and red cabbage (e, f); during spontaneous (■) and controlled fermentation (□). Each value is mean ± standard deviation of three measurements. *Significant difference from initial time for controlled fermentation; #significant difference from initial time for spontaneous fermentation; +significant difference between the type of fermentation-time (interaction).

antioxidant activity until five and ten days for the controlled and spontaneous fermentation, respectively; then, a second increase at the end of the process was observed (25 and 30 days) in both fermentations (Fig. 4d). In the controlled fermentation, the water extracts exhibited a significant increase in the antioxidant capacity on day 15. The values remained stable until day 30. However, the antioxidant capacity did not show significant variations in the spontaneous process (Fig. 4c). White cabbage did not exhibit significant differences in antioxidant activities between the two types of fermentation.

The antioxidant capacity of red cabbage showed a different pattern between the extracts (Fig. 4e vs 4f) during fermentation. In the methanol extracts, the antioxidant capacity increased significantly until day ten in spontaneous fermentation and then remained stable, while in controlled fermentation, the values slightly increased until the end of the experience (Fig. 4f). In the same way, the water extracts displayed a different pattern between controlled and spontaneous fermentation (Fig. 4e). In the controlled fermentation, the antioxidant activity slightly increased until the ten days and remained stable at the end of the process, while the spontaneous fermentation did not exhibit significant differences throughout the assay. Controlled fermentation of red cabbage showed an antioxidant activity higher than spontaneous process; in the water extracts, the differences were detected in the intermediate stage of the assay, while methanol extracts were on day 30.

4. Conclusions

In the present study, five autochthonous starters were selected among 120 isolated strains from cabbages spontaneous fermentation based on their technological properties (salt and pH acid tolerance, phenolics resistance, exopolysaccharide production, tannase, and pectinase activity) and further identified through molecular methods. Controlled fermentation of three *Brassica* vegetables was carried out by a two-steps method and, the results obtained suggest that this process exhibits advantages compared with spontaneous fermentation. The sharp decrease of pH during the first days in the controlled fermentation guarantees safety and shelf life of the final product preventing pathogens growth. Furthermore, both fermentations processes promoted changes in phenolic content and antioxidant activity which depended on type of fermentation and vegetable used. Regarding antioxidant activity, results obtained suggested that in comparison with spontaneous fermentation, the use of the selected mixed starter culture conducted to products with high antioxidant capacity. Controlled fermentation exhibited higher antioxidant capacity than spontaneous fermentation in chinese and white cabbage, regardless of the type of extract. Then, the controlled fermentation of red cabbage reached an antioxidant capacity greater than or equal to that of the spontaneous one, depending on the extraction conditions. However, red cabbage exhibited higher antioxidant capacity in comparison with chinese and white cabbage, regardless of

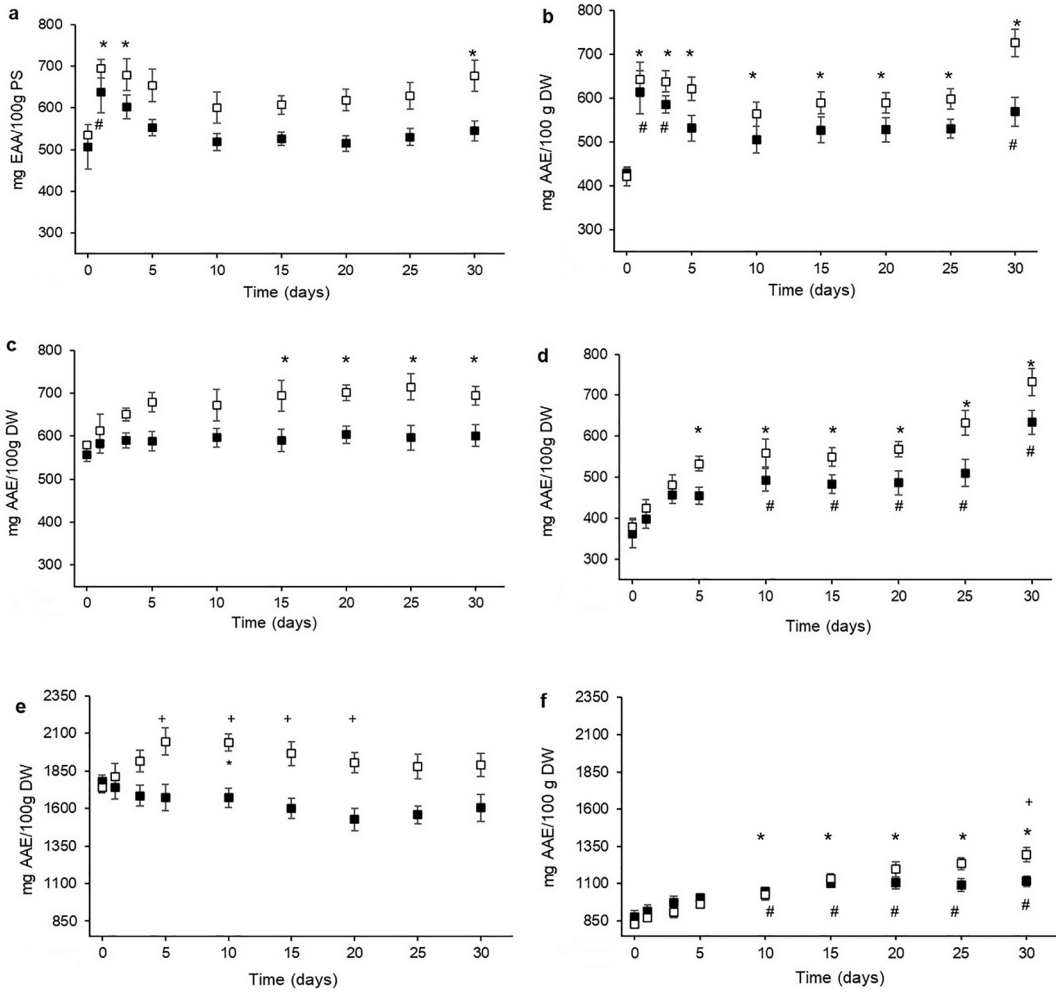

Fig. 4. Antioxidant activity (CUPRAC assay) in extracts (water (a, c, e) and methanol (b, d, f) of chinese cabbage (a, b), white cabbage (c, d) and red cabbage (e, f); during spontaneous (■) and controlled fermentation (□). Each value is mean ± standard deviation of three measurements. *Significant difference from initial time for controlled fermentation; #significant difference from initial time for spontaneous fermentation; +significant difference between the type of fermentation-time (interaction).

the type of fermentation and extraction conditions.

CRediT authorship contribution statement

Romina B. Parada: Conceptualization, Methodology, Software, Validation, Formal analysis, Investigation, Writing – review & editing, Visualization. **Emilio Marguet:** Conceptualization, Methodology, Formal analysis, Investigation, Writing – original draft. **Carmen Campos:** Conceptualization, Software, Formal analysis, Writing – review & editing. **Marisol Vallejo:** Conceptualization, Methodology, Writing – review & editing, Visualization.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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