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Oenococcus oeni allows the increase of antihypertensive and antioxidant activities in apple cider

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ABSTRACT

This study aimed to investigate the impact of the malolactic fermentation (MLF) carried out by *Oenococcus oeni* on antihypertensive and antioxidant activities in cider. The MLF was induced using three strains of *O. oeni*. The modification in phenolic compounds (PCs) and nitrogen organic compounds, antioxidant, and antihypertensive activities were determined after MLF. Among the 17 PCs analyzed caffeic acid was the most abundant compound and phloretin, (−)-epicatechin, and myricetin were detected only in malolactic ciders, however, (−)-epigallocatechin was not detected after MLF. The evaluation of nitrogen organic compounds revealed a drop in total protein concentration (from 17.58 to 14.00 mg N/L) concomitantly with a significant release of peptide nitrogen (from 0.31 to a maximum value of 0.80 mg N/L) after MLF. In addition, an extracellular proteolytic activity was evidenced in all MLF supernatants. The FRAP activity increased reaching a maximum of 120.9 μmol $FeSO_4$/mL and the ABTS radical-scavenging activity increased until 6.8 mmol ascorbic acid/L. Moreover, the angiotensin I-converting enzyme inhibitory activity reached a maximum value of 39.8%. The MLF conducted by *O. oeni* in ciders enables the increase of interesting biological activities and this finding could constitute a valuable tool to add value to final product.

1. Introduction

Cider is a traditional alcoholic beverage resulting from the alcoholic fermentation (AF) of apple juice by yeasts and, sometimes, from malolactic fermentation (MLF) carried out by lactic acid bacteria (LAB) [1]. In traditional (spontaneous) fermentations, non-*Saccharomyces* yeasts such as *Kloeckera*, *Candida*, *Pichia* and *Hansenula*, are at high cell density during the first days of fermentation, however, the genus *Saccharomyces* is usually the most important during AF [2]. In this sense, *Saccharomyces cerevisiae* with greater tolerance to ethanol, becomes dominant and maintains its activity until the end of fermentation [3,4]. In cider, *Lactobacillus* and *Oenococcus* were described as the predominant genera during spontaneous MLF, with *Leuconostoc* and *Pediococcus* being found in a low proportion [5,6]. The genus *Oenococcus* (from the Greek oinos = wine) currently comprises four species: *O. oeni*, *O. kitaharae*, *O. alcoholitolerans* and *O. sicerae*, which was recently isolated from French ciders [7]. Among all known LAB species, *O. oeni* is the main species found in cider cellars [5] and the most studied species especially for its ability to perform MLF in the hostile environment

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represented by wines and ciders (low nutrient availability, low pH, and high ethanol content) [8]. During MLF, L-malic acid is metabolized into L-lactic acid, a desirable process that improves the organoleptic characteristics of ciders, reducing their acidity and at the same time increasing their microbiological stability [9]. Additionally, previous studies have shown that the antioxidant properties of ciders are modified after the MLF [10], and similar behavior was observed in wines [11]. These modifications have been mainly attributed to changes in the phenolic profile of these beverages [12,13]. Phenolic compounds (PC) are extensively studied in fermented beverages for two main reasons, on the one hand, because they influence the sensory and organoleptic characteristics (aroma, flavor, astringency) and on the other hand, because of their multiple health benefits (antioxidants, anticarcinogenic, preventives of coronary diseases) [4]. The kind and concentration of PCs in ciders depend on factors including the apples varieties used, and the processing of the raw material to extract the juice [14]. In addition, it is known that microbial species involved in fermentation and inoculation methods have an important impact on the PCs in cider [10,14,15]. However, other biological activities were related to the presence of bioactive peptides released in the fermentative process. During fermentation, the proteins of the raw materials can be hydrolyzed to peptides with interesting biological activities due to the presence of proteolytic microorganisms [16]. There is evidence that *O. oeni* has developed several adaptive mechanisms to survive in the harsh conditions found in ciders and wines such as the expression of an extracellular proteolytic activity that enables the release of small peptides and free amino acids [17]. In this sense, the antihypertensive activity in wines has been related to the presence of peptides with angiotensin I converting enzyme inhibitory activity [18]. In the same way, Apud et al. [19] revealed an increase in the antihypertensive activity related to modification of peptidic composition by *O. oeni* metabolism in wine. In cider production, nitrogenous compounds come mainly from apples in the form of proteins, peptides, amino acids, and ammonium ions. Nitrogenous compounds can be a limiting factor for microbial growth and are one of the main components that influence the production of aromas, biogenic amines, formation and stabilization of foams and the stability of the final product [20–25]. However, nitrogenous compounds have not been explored in ciders yet regarding their involvement in biological activities.

In this work, modifications in the content of PCs and nitrogenous compounds were evaluated in cider. Additionally, changes in biological activities, such as antioxidant and antihypertensive activities after MLF carried out by three *O. oeni* strains were also studied.

To the best of our knowledge, this is the first investigation in Argentina about the influence of MLF in ciders and that reveals the ability of native *O. oeni* strains to enhance the beneficial properties of this popular beverage.

2. Materials and methods

2.1. Microorganisms, culture media, and inoculum preparation

A mixture of Red Delicious (75%) and Granny Smith (25%) apple varieties was used to obtain apple juice (AJ) using a juice extractor. The AJ was filtered through a Whatman filter paper No. 2 and pasteurized for 30 min at 63 °C. A total volume of 1.7 L of pasteurized juice was obtained. An active dried commercial preparation of *Saccharomyces cerevisiae* EC1118 (Lalvin, Danstar Ferment AG, Denmark) was rehydrated (0.2 g of yeast per liter of medium) in sterile YPD broth pH 5.0, at 30 °C for 24 h. The inoculum was obtained by transferring an aliquot of active yeast culture (8 mL) to pasteurized AJ (72 mL) and grown under aerobic conditions with continuous agitation (250 rpm) at 30 °C for 48 h. Three different *Oenococcus oeni* strains were used for malolactic fermentation (MLF). RAM10 and RAM11 were isolated from wines in Tucumán, Argentina [26] and the commercial VP41 strain was obtained from Lallemand (LALLFERM S.A, Mendoza, Argentina). *O. oeni* strains were stored at −20 °C in De Man Rogosa Sharpe medium (Oxoid Ltd., London, England) supplemented with 30% (v/v) glycerol. Strains were first activated in MLO broth [27] with 5% (v/v) ethanol at 30 °C until the exponential phase of growth ($OD_{560} = 0,6$). Active cultures were centrifuged (5000 *g*, 10 min) and the residual medium was removed by washing the pellet with 0.9% NaCl. Pellets were resuspended in an aliquot of filtered and pasteurized cider (apple juice after the alcoholic fermentation was completed), and then an adequate volume of this suspension was used as inoculum in cider to carry out MLF.

2.2. Small-scale fermentation conditions

2.2.1. Alcoholic fermentation

AF assays were carried out in 2.0 L capacity Erlenmeyer flasks. All fermentations were conducted in duplicate and treated independently. A volume of 1.2 L of pasteurized AJ was inoculated with an aliquot of the *S. cerevisiae* inoculum described above (Section 2.1), and the yeast concentration was adjusted to 10^6 cfu/mL. The AF was performed under static conditions at 18 °C. Estimation of the sugar consumption and AF progress were monitored by daily determination of the weight loss of the fermentation system due to CO_2 release [28]. After 16 days of incubation, the specific gravity was 1,006, so the AF was considered completed. Finally, the cider obtained was centrifuged at 10,000 *g*, filtered through a cellulose nitrate membrane (0.45 μm), and pasteurized for 30 min at 63 °C. In the present study, this base cider is referred to as "AF cider" and it was used for analytical determinations and MLFs.

2.2.2. Malolactic fermentation

A volume of 100 mL of AF cider was inoculated with the different strains of *O. oeni* separately at a cell concentration of 10^6 cfu/mL. Fermentations with each *O. oeni* strain were carried out in pre-sterilized 100 mL bottles at 18 °C and under static conditions. All MLFs were carried out in duplicate, and each replicate was treated independently. Every 24 h, samples were taken for further analysis. An aliquot was used for bacterial cell counts. In the remaining volume, cells were harvested by centrifugation at 5000 *g* for10 min and supernatants were collected, filtered (0.22 μm filter, Millipore) and stored at −20 °C until usage. When residual malic acid was lower than 0.4 g/L, the MLF was considered completed. In our study samples with AF plus MLF are referred to as "MLF cider".

2.3. Viability and fermentative capacity of the O. oeni strains

Enumeration of *O. oeni* was performed by the standard plate-counting method on MLO agar, pH 5.5. Plates were incubated under anaerobic conditions at 30 °C for 5 days. The progress of MLF was controlled by monitoring malic acid consumption. The concentration of malic acid was determined with the Boehringer enzymatic kit (R-BIOPHARM AG, Darmstadt, Germany) according to the manufacturer.

2.4. Chemicals and reagents

Bradford reagent was obtained from Bio-Rad (California, USA). Acetic acid, trichloroacetic acid (TCA), tin chloride, and cadmium chloride were purchased from Merck (Darmstadt, Germany). Ninhydrin, 2-methoxyethanol, L-leucine, 2,2-dipheny/L-picrylhydrazyl (DPPH), 2,4,6-tris-(2-pyridyl)-*s*-triazine, 2,2′-Azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS), ascorbic acid (AA), ferric chloride, d (+)-Glucose, bovine serum albumin (BSA), sodium acetate, sodium chloride, 3,5- dinitrosalicylic acid (DNS), acetone, methanol, ethanol, ferrous sulfate heptahydrate, citric acid, sodium citrate, hippuryl-histidyl-leucine, Angiotensin Converting Enzyme (ACE), hydrochloric acid, ethyl acetate, 3-hydroxytyrosol (≥99.5%), caftaric acid (≥97%), (−)-epigallocatechin (≥95%), (+)-procyanidin B1 (≥90%), (+)-catechin (≥99%), procyanidin B2 (≥90%), (−)-epicatechin (≥95%), caffeic acid (99%), coumaric acid (99%), quercetin hydrate (95%), quercetin 3-β-d-glucoside (≥90%), kaempferol-3-glucoside (≥99%), myricetin (≥96%), naringin (≥95%), phloridzin dehydrate (99%) and phloretin (≥99%), were obtained from Sigma–Aldrich (St. Louis, MO, USA). The standard of 2-(4-hydroxyphenyl) ethanol (tyrosol) (≥99.5%) was obtained from Fluka (Buchs, Switzerland). The acetonitrile (MeCN), ethanol, methanol, and formic acid (FA) were of HPLC-grade and acquired from Mallinckrodt Baker (Inc. Pillispsburg, NJ, USA). Ultrapure water was obtained from a Milli-Q system (Millipore, Billerica, MA, USA).

2.5. Analytical methods

AJ, AF cider, and MLF ciders samples were used for analytical determinations. The pH was measured using a 744-pH meter (Metrohm, Switzerland). Water-soluble solids (Brix) were measured with an ICSA OPTIC refractometer (Model REF103, Argentina). Reducing sugars (RS) were estimated using the DNS method by Miller [29] using glucose as standard. Ethanol content was determined with an Anton Paar DMA 35 basic density meter (Graz, Austria).

2.6. Total phenolic compounds (TPC)

To determine the TPC, the Folin-Ciocalteu assay was used. This technique is based on the reaction of the PC with the Folin-Ciocalteu reagent, at basic pH, which gives rise to a blue color that can be determined spectrophotometrically at 740 nm [30]. This reagent contains a mixture of phosphotungstic acid and phosphomolybdic acid which are reduced in alkaline medium in the presence of PC. The yellow reagent, when reduced by the phenolic groups, gives rise to a complex of intense blue color, whose intensity is proportional to the PC concentration of the tested sample.

To 100 μL of each sample, 100 μL of the Folin-Ciocalteu reagent (Sigma) was added. Shake and incubate for 2 min at room temperature. Next, 800 μL of 5% (w/v) Na_2CO_3 were added, incubated for 20 min at 40 °C and the OD was determined at $\lambda = 740$ nm in a Jenway 7305 spectrophotometer.

The TPC content was estimated by comparison with OD values obtained with different concentrations of gallic acid solutions (12.5 to 400 mg/L) as standard. The determinations were performed in triplicate and the results were expressed as mg/L of gallic acid equivalents (GAE).

2.7. Flavonoid content (FC)

To determine the FC in the samples, the methodology described by Rodríguez Vaquero et al. [31] was used. The method is based on the ability of formaldehyde to react with the hydroxyl groups of flavonoid compounds, forming water-insoluble condensation molecules. 5 mL of each sample was treated with 5 mL of diluted HCl (1:3) and 2.5 mL of a formaldehyde solution (8 mg/mL). It was allowed to precipitate for 24 h, centrifuged at 8000 rpm for 5 min and the content of non-flavonoid compounds (phenolic acids) was determined in the supernatant using the Folin-Ciocalteu reagent as previously described. By difference between the content of TPC and that of phenolic acids, the content of FC present in each sample was obtained. The determinations were carried out in triplicate and the results were expressed as mg GAE/L.

2.8. Analysis of phenolic compounds by HPLC

A liquid chromatography method coupling diode-array and fluorescence detectors (DAD and FLD, respectively) developed by Ferreyra et al. [32] was used. Seventeen PCs present in samples were simultaneously quantified. An HPLC-DAD-FLD (Dionex Ultimate 3000 system, Dionex Softron GmbH, Thermo Fisher Scientific Inc., Germering, Germany) and a reversed-phase Kinetex C18 column (3.0 mm × 100 mm, 2.6 μm; Phenomenex, Torrance, CA, USA) were used. The mobile phases were an aqueous solution of 0.1% FA (eluent A) and MeCN (eluent B). The following gradient was used: 0–1.7 min, 5% B; 1.7–10 min, 30% B; 10–13.5 min, 95% B; 13.5–15 min, 95% B; 15–16 min, 5% B; 16–19, 5% B. The total flow rate was set at 0.8 mL/min and the column temperature at 35 °C. Samples

(5 μL) were filtered and degassed prior to injection. The conditions for DAD and FLD detectors were as follows: the analytical flow cell for DAD was set to scan from 200 nm to 400 nm and different wavelengths (254, 280, 320 and 370 nm) were used according on the maximum absorbance of analytes. For FLD, an excitation wavelength of 290 nm and a monitored emission responses of 315, 360 and 400 nm were used depending on the targeted analytes. The retention times of compounds in samples with those of standards was the way of identification of each PC and quantification was done by an external calibration with pure standards. All the samples were analyzed in the triplicate.

2.9. Total protein analysis

The Bradford assay was used to determine the protein concentration. The Bradford reagent (previously diluted 1:5 with water) was added to 0.05 mL of sample. A calibration curve was constructed using BSA as standard and absorbance was measured at 595 nm with a Jenway 7305 spectrophotometer (Staffordshire, UK) after 20 min of incubation at 20 °C. To calculate the protein nitrogen concentration (mg N/L) in the samples, the molecular weight of the protein standard (66.432 g/mol) and the nitrogen atoms contained in the molecule (10.276 g/mol) were considered.

2.10. Modification of free amino acids and peptides

Modifications of free amino acids and peptides were estimated according to Alcaide-Hidalgo et al. [18]. L-leucine was used as standard for the calibration curve with a concentration range of 0.06 to 0.5 mM. For calculations, the molecular weight of leucine (131.17 g/mol) and the number of nitrogen atoms present in the molecule (14 g N for every 131.17 g of leucine) were considered. Results are expressed as mg of free amino nitrogen/L (mg aN/L) and mg of peptide nitrogen/L (mg pN/L).

2.11. Extracellular proteolytic activity of O. oeni determination

Aliquots of the supernatants (0.25 mL) obtained at different times of each MLF assay were considered as enzyme solution and the pasteurized AJ (0.20 mL) as the protein substrate. Proteolysis was conducted in 0.65 mL of 0.05 M citrate buffer, pH 5.0. After 1h of incubation at 30 °C, the reaction was stopped by adding 24% TCA (0.65 mL). The samples were then refrigerated for 15 min at 4 °C, centrifuged at 5000 *g* for 5 min, and the supernatants were used for determination of free amino acid and peptides released from AJ proteins according to procedures descripted previously (Section 2.10). In all cases, controls were obtained by precipitation with TCA immediately before incubation. Proteolytic activity is expressed as the concentration of amino acid and peptide nitrogen released (mg N/L).

2.12. Biological activities determination in the AJ, AF cider, and MLF ciders

2.12.1. DPPH radical-scavenging activity

Antiradical activity was determined using the procedure described [12] with some modifications. A volume of 0.25 mL of sample previously diluted 1:5 with methanol was added to 1 mL of a methanol solution of 0.06 mM DPPH and it left to stand in the dark. Absorbance was measured at 517 nm at the beginning and after 30 min [33]. Absorbance of the samples was related to the vitamin C equivalent antioxidant capacity (VCEAC) using a standard curve of ascorbic acid (AA). The results are expressed as VCEAC (mmol AA/L).

2.12.2. ABTS radical-scavenging activity

The ABTS assay was used to determine the effectiveness of antioxidants in the samples in reducing the ABTS radical cation according to the procedures described by Rivero et al. [34]. Briefly, an aqueous solution of ABTS (7 mM) was incubated with potassium persulfate in the dark by 16 h to generate the radical cation. Then, the concentration of this solution was adjusted with ethanol to an absorbance value of 0.70 at 734 nm. The reaction mixture was constituted by 0.01 mL of ascorbic acid (standard) or the samples and 0.19 mL ABTS radical solution, and it was incubated for 6 min at 25 °C. The decrease of absorbance was monitored at 734 nm. The results are expressed as VCEAC (mmol AA/L).

2.12.3. Ferric reducing antioxidant power (FRAP)

This assay measures the formation of a colored Fe (II)-tripyridyltriazine complex from colorless oxidized Fe (III) as a result of electron-donating antioxidants [35]. The working FRAP reagent was prepared according to Ref. [12]. A volume of 900 μL of the FRAP reagent was mixed with 30 μL of a previously 1:2 diluted sample. The assay was carried out at 37 °C for 15 min and the absorbance was measured at 593 nm. A standard curve was constructed using a Fe (II) sulfate solution (10 to 1000 μM) and the results are expressed as the equivalent of μmoles Fe^{2+} per mL of sample (μmol $FeSO_4$/mL). All determinations for antioxidant activity were carried out in triplicate.

2.12.4. In vitro angiotensin I converting enzyme (ACE) inhibition

The methodology described by Alcaide-Hidalgo et al. [18] was used to calculate the ACE inhibitory activity (ACEI) of the samples. To determine the capacity of the samples (inhibitors) to inhibit ACE, the hippuric acid released from hippuryl-histidyl-leucine (substrate) was quantified. Briefly, a tube "A" without sample was prepared, which allowed the reaction of the enzyme under optimal

conditions, a tube "B" in which the enzyme was inactivated by adding HCl before incubation (reaction blank), and tubes "C", which were supplemented with the samples used as inhibitor, substrate, and enzyme. All tubes were incubated at 37 °C for 80 min and then the reaction was stopped with HCl. After addition of ethyl acetate to the tubes, they were centrifuged at 3000 *g* for 10 min, in order to extract the organic phase containing the released hippuric acid. Samples were dried at 37 °C for 24 h, dissolved in distilled water and then the absorbance was read at 228 nm to quantify the hippuric acid formed. All determinations were carried out in triplicate. The percentage of enzyme inhibition was estimated with the following equation:

$$\%ACEI = 100 \times [A - C / A - B]$$

Where A, B, and C represent the average of the absorbances determined in each reaction tube.

2.13. Statistical analysis

Data from analytical determinations are the means of two independent experiments carried out in triplicate. One-way analysis of variance was applied to the experimental data. Variable means with statistically significant differences were compared using Tukey's test. The data of bacterial viability and malic acid consumption were analyzed using the Student's t-test. All statements of significance are based on a probability of 0.05.

3. Results and discussion

3.1. Alcoholic and malolactic fermentation

In the present study, AF carried out with a commercial *S. cerevisiae* strain on AJ produced a base cider with a final alcohol content of 5.2% (v/v). Similar values of ethanol were reported in the scientific literature [2,5], although some authors reported values of ethanol between 6.0% and 12% at the end of the AF in ciders [36]. The final alcohol content in ciders depends mainly on the content of fermentable sugars present in the apples and the temperature at which fermentation takes place [2]. In this sense, some apple varieties such as "Golden Russet", "Gloster" or "Idared" can reach high sugar concentrations that result in ciders with 12% alcohol [37,38]. Table 1 shows the basic physicochemical values for AJ and cider after AF and MLF. A slight decrease in malic acid concentration in the cider compared with the juice was observed, that could be related to the yeast capacity to metabolize this acid [37]. On the other hand, according to Budak et al. [39], most of the sugars present in AJ were consumed, dropping Brix and RS values at the end of AF due to yeast metabolism. Finally, the CO_2 released after 16 days of AF resulted in a 60 g weight loss of system (3.1 g/L CO_2/day).

MLF can be induced by simultaneous inoculation of yeast and bacteria (co-inoculation), inoculation during AF, and inoculation after completion of AF (sequential inoculation) [1]. When occurring simultaneously, yeasts can negatively affect LAB growth and thus MLF development. This damage is due to antagonism by nutrients, as well as by the toxicity of some metabolites produced by yeasts such as ethanol, organic acids, medium chain fatty acids and antimicrobial peptides [40]. For this reason, sequential inoculation presents advantages that include the absence of antagonistic interactions between yeast and bacteria, as well as a low residual sugar concentration, which reduces the risk of production of undesirable metabolites by heterofermentative LAB [41]. In this work, the inoculation with the three strains of *O. oeni* was carried out once the AF had finished. As shown in Fig. 1, the three *O. oeni* strains were able to growth and successfully conduct the MLF in cider. RAM10 and RAM11 strains consumed approximately 90% of the malic acid after 7 days, reaching a final residual concentration of 0.27 and 0.36 g/L, respectively. The VP41 strain reached the end of the MLF 5 days after inoculation consuming 98.9% of the total malic acid. The three strains showed the maximum increase in viable cell count during the first 24 h incubation, after this time a maintenance in viability around 10^8 cfu/mL was detected until final incubation time. Similarly, Reuss et al. [9] and Laaksonen et al. [42] reported the use of two *O. oeni* strains (MCW and VP41) from wine to conduct the MLF in cider. After the MLF an increase in the pH values and a decrease in the concentration of reducing sugars were observed.

The growth of the bacteria in the culture medium was determined by means of statistical analysis using the Student's t-test. The results showed a statistically significant increase in bacterial growth in the culture medium over time, indicating that the conditions provided by the medium were suitable for bacterial proliferation.

Table 1
Physicochemical values obtained from apple juice (AJ), cider after 16 days of alcoholic fermentation (AF cider) and MLF ciders.

Parameters	AJ	AF CIDER	MLF RAM10	MLF RAM11	MLF VP41
pH	3.96 ± 0.02	3.92 ± 0.02	4.26 ± 0.03	4.28 ± 0.03	4.30 ± 0.03
Malic acid (g/L)	4.07 ± 0.28	3.60 ± 0.21	0.274 ± 0.02	0.364 ± 0.02	0.04 ± 0.01
BRIX (%)	12.80 ± 0.02	4.00 ± 0.01	2.60 ± 0.01	2.60 ± 0.01	3.00 ± 0.01
RS (g/L)	110.1 ± 6.2	3.3 ± 0.4	1.8 ± 0.2	1.7 ± 0.2	2.6 ± 0.3

Values are the means of two independent experiments carried out in triplicate ± standard deviation (±SD).
RS: Reducing Sugars.

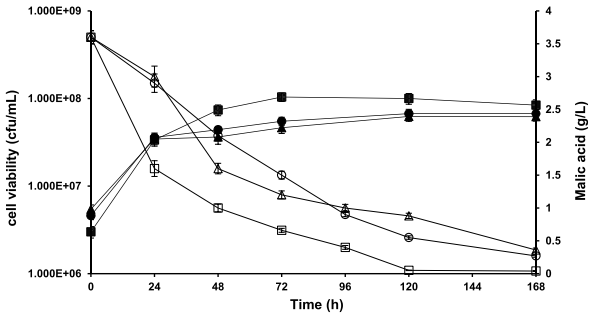

Fig. 1. Malic acid (open symbol) and cell viability (solid symbol) evolution during MLF. (○) *O. oeni* RAM10, (Δ) *O. oeni* RAM11, (□) *O. oeni* VP 41.

3.2. Modification of total phenolic and flavonoids after MLF

Table 2 shows the values obtained for TPC and FC corresponding to samples of AJ, AF cider and, MLF ciders. In our study, we observed a decrease in the TPC and FC after AF. Similar results were reported in ciders obtained from Dous Moen and Idared apple varieties [15,43]. On the other hand, an increase in TPC and FC was observed after MLF carried out with the three strains analyzed compared to AF. Similarly, Hernández et al. [44] reported that MLF increased the PCs concentration in wines. However, other authors reported a decrease in TPC and an increase in FC after MLF in cider carried out by *O. oeni* PG-16 strain [10]. In this work, the highest TPC and FC concentration values were obtained in cider fermented by VP41 strain (745.9 and 270.6 mg GAE/L, respectively). On the other hand, after MLF, the TPC values determined in ciders inoculated with RAM10 and RAM11 strains did not present significant differences ($P < 0.05$), and the RAM10 strain showed the lowest FC value.

3.3. Modifications of individual phenolic compounds after MLF

Table 3 shows 17 compounds analyzed that belong to the five families of PCs normally found in apple derivatives [4]. These were hydroxycinnamic acids (caffeic acid, caftaric acid, and *p*-cumaric acid), flavonols (quercetin and quercetin-3-glucoside, kaempferol-3-glucoside, and myricetin), flavan-3-ols (procyanidins B1 and B2, (+)-catechin, (−)-epicatechin, and (−)-epigallocatechin), volatile phenols (tyrosol and OH-tyrosol) and dihydrochalcones (phloretin and phloridzin). About the individual PCs, caffeic acid was the major compound, representing more than 90% of the PCs analyzed and its concentration was higher in MLF ciders. However, Laaksonen et al. [42] observed a decrease in the caffeic acid content after MLF. On the other hand, in a study carried out with 8 Asturian ciders, this compound was found in only two of them with concentrations lower than 12 mg/L [45]. Concerning volatile phenols, tyrosol increased after the MLF carried out with the RAM10 and VP41 strains. Although tyrosol is produced by yeast from tyrosine, other authors also reported its increase after MLF [44]. The values obtained in our work for procyanidins B1 and B2 were significantly lower than those obtained by Suárez et al. [45] in Spanish ciders. However, values of (+)-catechin were similar to those reported for these authors. On the other hand, (−)-epigallocatechin decreased drastically after AF; on the contrary [15], reported an increase of this compound after AF. Our results show that (−)-epigallocatechin was the only PC that was not detected after the MLF performed by the three strains tested. However, (−)-epicatechin was detected only after MLF. Regarding the flavonols, the concentrations of quercetin-3-glucoside did not change after the fermentations. On the other hand, its aglycone, quercetin, was only detected after AF and MLF, so its presence may be due to the activity of microbial glycolytic enzymes [46]. Similarly, a study carried out with different apple cultivars showed an increase in free quercetin after AF [42]. On the contrary, Li et al. [10] observed a decrease in both quercetin and its glycosides after fermentation. García-Ruiz et al. [47] reported that MLF affects the phenolic composition of wine, reducing the contents of anthocyanins and total polyphenols, however, Hernandez et al. [44] showed that MLF also gives rise to some new PCs not

Table 2
Phenolic and nitrogen compounds modification in cider production. Proteolytic activity of *Oenococcus oeni* RAM10, RAM11 and VP41 strains.

	AJ	AF cider	MLF ciders		
			RAM10	RAM11	VP41
TPC (mg GAE/L)	772.4a±32.6	708.1d ± 25.1	715.3c±30.7	720.4c±29.6	745.9b ± 30.1
FC (mg GAE/L)	250.9b ± 17.2	204.1d ± 14.4	222.5c±18.1	242.8b ± 20.7	270.6a±16.6
Proteins (mg N/L)	28.93a±1.7	17.58b ± 0.88	14.86d ± 0.74	14.0c ±0.6	14.01c±0.6
P (mg pN/L)	5.65a±0.48	0.31e±0.025	0.59d ± 0.03	0.68c ±0.03	0.8b ± 0.04
FAA (mg aN/L)	19.28a±1.66	0.69c±0.053	0.88b ± 0.044	0.69c±0.034	0.92b ± 0.036
PA (mg N/L)	–	nd	2.3c±0.11	5.86a±0.23	4.06b ± 0.21

Values are the means of two independent experiments carried out in triplicate ± standard deviation (±SD). Values with different letters (a–e) in the same row are significantly different according to the Tukey's test ($p < 0.05$).
MLF ciders = ciders after 7 days of malolactic fermentation, AJ = apple juice, AF cider = cider after alcoholic fermentation, TPC = total phenol compounds and FC = flavonoid content as mg gallic acid equivalents per liter, P = peptides as mg peptide nitrogen per liter, FAA = free amino acids as mg amino nitrogen per liter, PA = maximum proteolytic activity, nd = not detected.

Table 3
Modification of individual phenolic compounds during cider production.

Sample	AJ		AF cider		MLF ciders					
					RAM10		RAM11		VP41	
Analyte	mg/L	SD	mg/L	SD	mg/L	SD	mg/L	SD	mg/L	SD
OH-Tyrosol	0.91a	0.07	0.17d	0.01	0.32b	0.03	0.28c	0.02	0.27c	0.02
Tyrosol	1.38d	0.11	6.27c	0.50	7.17b	0.57	6.83b	0.55	7.28a	0.58
Procyanidin B1	1.15c	0.09	2.56a	0.20	2.95a	0.24	2.86a	0.23	2.34a	0.19
(+)-catechin	1.37d	0.11	2.27a	0.18	2.28a	0.18	2.4a	0.19	1.92b	0.15
Procyanidin B2	3.57a	0.29	3.46a	0.28	4.05a	0.32	3.92a	0.31	3.22a	0.26
(−)-epicatechin	n.d	–	n.d	–	15.66a	1.25	15.29a	1.22	14.43b	1.15
Quercetin-3-glucoside	2.08a	0.17	2.22a	0.18	1.99a	0.16	1.93a	0.15	2.15a	0.17
Kaempferol-3-glucoside	3.65b	0.29	5.05a	0.40	4.86a	0.39	4.78a	0.38	4.63a	0.37
(−)-epigallocatechin	66.82a	5.35	6.44b	0.52	n.d	–	n.d	–	n.d	–
Naringin	2.32b	0.19	2.85a	0.23	2.66a	0.21	2.39a	0.19	2.88a	0.23
Phloridzin	7.17b	0.57	10.04a	0.80	5.28c	0.42	4.93c	0.39	4.31d	0.34
Phloretin	n.d	–	n.d	–	0.74c	0.06	0.73c	0.06	0.8c	0.06
Caftaric acid	1.69a	0.14	0.47b	0.04	0.46b	0.04	0.44b	0.04	0.18c	0.01
Caffeic acid	480.1b	38.41	463.23b	37.06	504.82a	40.39	500.72a	40.06	507.64a	40.61
p-coumaric acid	0.2b	0.02	0.17b	0.01	n.d	–	0.66a	0.05	n.d	–
Myricetin	n.d	–	nd	–	0.24a	0.02	0.23a	0.02	0.22a	0.02
Quercetin	n.d	–	0.12b	0.03	0.30a	0.02	0.29a	0.02	0.29a	0.02
Total sum	572.4a	46.2	505.3c	40.2	553.7b	41.5	548.6b	41.0	552.5b	41.1

Values are the means of two independent experiments carried out in triplicate ± standard deviation (±SD). Values with different letters (a–d) in the same row are significantly different according to the Tukey's test ($p < 0.05$).
MLF ciders = ciders after 7 days of malolactic fermentation carried out with *Oenococcus oeni* RAM10, RAM11 and VP41 strains, AJ = apple juice, AF cider = cider after alcoholic fermentation, nd = not detected.

detected in the initial wine. In this sense, malolactic ciders were similar in terms of polyphenol content, except for the presence of *p*-coumaric acid only in the cider obtained with the RAM11 strain. This is because of, during MLF, it has been reported that *O. oeni* strains with cinnamoyl esterase activities can hydrolyze hydroxycinnamic esters, for example, coutaric acid, increasing the corresponding free forms, such as *p*-coumaric acid [48]. Finally, the most notable changes after MLF include the presence of phloretin, (−)-epicatechin and myricetin in malolactic ciders, which is consistent with previous studies [42].

Several authors have studied the effect of some PCs on *O. oeni* [49,50] finding that the growth of this microorganism can be affected by PCs in different ways, depending on its type and concentration. Among the different PCs, it was reported that hydroxycinnamic acids inhibited the growth of *O. oeni*, with *p*-coumaric acid showing the greatest inhibitory effect on growth and survival. In this work, the caffeic acid present in the ciders was found in concentrations higher than 500 mg/L; however, the viability of the three strains studied remained at values higher than 10^7 cfu/mL until the end of the MLF, therefore that this acid would not present an inhibitory effect under the tested conditions. Figueiredo et al. [51] also reported that flavonoids such as quercetin and kaempferol exert an inhibitory effect on *O. oeni* at concentrations higher than 10 mg/L. The values detected in the ciders obtained in this work for these compounds did not exceed 5 mg/L, so we could not observe any negative effect on the 3 strains of *O. oeni* tested. These results allow us to hypothesize that the phenolic composition of the ciders obtained did not negatively influence the development of *O. oeni* or the concretion of MLF.

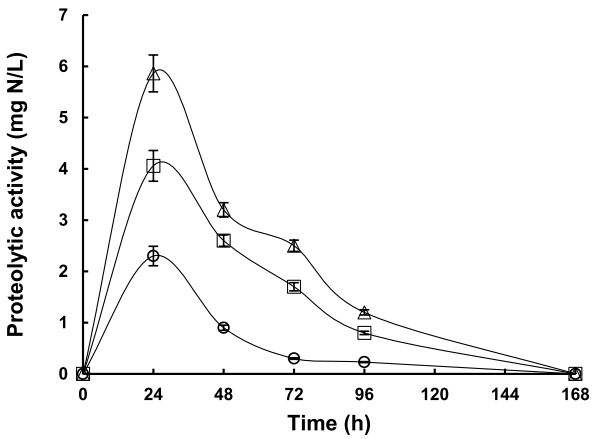

Fig. 2. Proteolytic activity of MLF supernatants of (○) *O. oeni* RAM10, (Δ) *O. oeni* RAM11, (□) *O. oeni* VP41.

3.4. Modification of nitrogen compounds concentration during cider production. Proteolytic activity of O. oeni

Table 2 shows that *S. cerevisiae* consumes most of the nitrogen compounds present in AJ, a behavior caused by the fact that nitrogen is the main limiting nutrient during AF [52]. Among these compounds, amino acids are essential nutrients for yeast growth and their concentration in cider depends on different factors such as the type of apple and yeast strain used in the process, yeast autolysis, and aging time [53]. It has also been reported that several amino acids can be intermediates or precursors of volatile compounds and biogenic amines which influence the aroma and quality of the cider [4,53]. After AF, in an environment with few available nutrients, the three strains of *O. oeni* studied in this work were able to release peptides and free amino acids during MLF. The results show that after 7 days of MLF the total protein content decreased in all cases, while an increase in the amino acid and peptide nitrogen content was observed. In this sense, several authors reported a release of proteolytic enzymes into the extracellular medium by *O. oeni* under similar nutritional stress conditions [17]. Strains RAM11 and VP41 showed the highest decrease in protein concentration followed by an increase in peptide nitrogen concentration. Based on these results, the proteolytic activity in the MLF supernatants was examined. As seen in Fig. 2, proteolytic activity was detected in the supernatants of all three strains assayed. This activity was highest after 24 h of MLF, which corresponds to the end of the exponential growth phase of the bacteria (Fig. 1). Our results show that the three assayed strains differ in their proteolytic capacities against to the same substrate, and even though the RAM11 strain showed the highest proteolytic activity, the VP41 strain achieved a greater release of peptides and amino acids after MLF (Table 2). A previous study postulated that a high availability of peptides is directly related to a more competitive adaptation of *O. oeni* to an environment poor in nitrogen compounds [52]. In effect, the faster MLF and the higher viable cell count observed with the VP41 strain could be related to a higher availability of peptides and free amino acids.

3.5. Biological activities

DPPH, ABTS, FRAP, and ACEI values are shown in Table 4. After the AF, the DPPH and ACEI activities did not change with respect to those obtained with AJ, but instead, a decrease in ABTS and FRAP activities was observed. During MLF, DPPH radical scavenging activity of all analyzed cider samples remained unchanged, however, the values obtained were similar to that reported for Asturian ciders [12]. On the other hand, an increase in ABTS, FRAP and ACEI activities was observed in MLF ciders carried out by the three strains assayed. The highest antioxidant activities were obtained in presence of the VP41 strain, which produced an increase by more than 60 and 15% in the ABTS and FRAP activities, respectively. On the other hand, the antioxidant activities in ciders inoculated with RAM10 and RAM11 did not show significant differences. In this sense, an increase in antioxidant activities post-MLF were also reported in wines [19]. On the other hand, the only study that investigated the modification of biological activities after the MLF in ciders demonstrated that the increase in the ABTS, DPPH and FRAP activities were linked to the modification in PCs content [10]. Several studies have reported that some LAB strains have antioxidant activity by themselves due to the chelation capacity of metal ions, the elimination of reactive oxygen species, the inhibition of some enzymes and because they present reducing activity [54–56]. Regarding *O. oeni*, Su et al. [57] reported that this microorganism presents antioxidant activity that could be attributed to the reduction capacity of the ferric ion, the scavenging capacity of the DPPH radical or the scavenging capacity of reactive oxygen species. These authors also observed that the antioxidant activity depends on the strain and the culture medium. In this sense, our results showed that the ciders obtained with the different *O. oeni* strains tested presented different antioxidant activities.

The higher antioxidant activity detected after MLF could be related to the increase in flavonoid aglycones (quercetin, and phloretin) than have greater antioxidant properties than their glycosides (quercetin-3-glucoside and phloridzin) as previously postulated by Schubertová et al. [58]. In the same way, the increase in tyrosol concentration, compound with high antioxidant capacity [59], could also be associated to the enhancement of this property in cider after MLF. Caffeic acid, the main compound in all the samples, has been reported as a powerful antioxidant and this activity increases in combination with other compounds such as caftaric acid (also detected in MLF ciders)[60]. The increase in antioxidant activity after the modification of PC by BAL has also been reported in AJ. A study showed that apple juice fermented with *Lactobacillus plantarum* ATCC14917 improved antioxidant capacity by increasing the contents of quercetin, phloretin, and 5-O-caffeoylquinic acid during 24 h of fermentation [61]. In conclusion, the improved antioxidant activity after MLF would be mainly related to the PCs modification. Finally, ACEI activity significantly increased to an average value of 26.8% for MLF ciders, being the highest value that was obtained after MLF with RAM11 strain. To date, this is the first report that demonstrates the presence of angiotensin I converting enzyme inhibitory activity in ciders. Similar results were reported in wine, attributable to a proteolytic strain of *O. oeni*, that increased ACEI activity due to peptide release from the protein and polypeptide fraction of wines [19]. Other authors demonstrated that both, yeasts and LAB play an important role in the ACEI activity due to peptide release during the manufacture of red wines aged on lees [18]. ACEI activity is generally attributed to the presence of peptides [62,63]. However, previous studies have shown that some PCs, such as flavonoids and phenolic acids, exhibit the ability to inhibit different enzymes, including ACE [64–67]. In our work, the ACEI activity observed could also be related to the presence of epicatechin and phloretin (evidenced only after MLF). ACEI activity of these compounds was previously reported by Actis-Goretta et al. [68] and Al Shukor et al. [69].

This study demonstrated an increase in biological activities (ABTS, FRAP, and ACEI) after MLF that occurs concomitantly with an increase in TPC, FC, some individual polyphenols, and peptides in the medium. Furthermore, to the best of our knowledge, this is the first study that evidenced the presence of ACEI activity in ciders and the ability of *O. oeni* to increase this activity during MLF. Based on the results obtained and taking into account that many oenological species have presented probiotic characteristics [57,70,71] the native strains RAM10 and RAM11 are excellent candidates to evaluate their probiotic potential in future research.

Table 4
Biological activities modification after malolactic fermentation (MLF).

		DPPH (VCEAC)	ABTS (VCEAC)	FRAP (μmol $FeSO_4$/mL)	ACEI (%)
MLF ciders	AJ	3.63a±0.14	9.3a±0.48	159.9a±9.2	14.1d ± 0.8
	AF cider	3.72a±0.15	4.1d ± 0.28	101.7d ± 2.2	14.3d ± 0.8
	RAM10	3.72a±0.15	5.5c±0.24	109.1c±4.1	18.8c±1.1
	RAM11	3.66a±0.12	5.2c±0.21	108.4c±3.6	39.8a±2.8
	VP41	3.67a±0.12	6.8b ± 0.27	120.9b ± 6.2	21.7b ± 0.9

Values are the means of two independent experiments carried out in triplicate. Values with different letters (a–d) in the same column are significantly different according to the Tukey's test ($p < 0.05$).
AJ = apple juice, AF cider = cider after alcoholic fermentation, MLF ciders = ciders after 7 days of malolactic fermentation carried out with *Oenococcus oeni* RAM10, RAM11 and VP41 strains, VCEAC = vitamin C equivalent antioxidant capacity in mmol per liter of ascorbic acid.

4. Conclusions

Our results show that MLF is a desirable process that enhances the beneficial biological activities of this drink beyond the already known organoleptic and technological properties, so these findings will be useful to improve the attractiveness of ciders for consumers.

Statements of declarations

Author contribution statement

Irina Kristof: Conceived and designed the experiments; Performed the experiments; Analyzed and interpreted the data; Wrote the paper.

Silvana Cecilia Ledesma, Gisselle Raquel Apud: Performed the experiments; Analyzed and interpreted the data.

Nancy Roxana Vera: Contributed reagents, materials, analysis tools or data; Wrote the paper.

Pedro Adrián Aredes Fernández: Conceived and designed the experiments; Contributed reagents, materials, analysis tools or data; Wrote the paper.

Data availability statement

Data included in article/supplementary material/referenced in article.

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.

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Appendix A. Supplementary data

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