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Optimised method for the analysis of phenolic compounds from caper (*Capparis spinosa* L.) berries and monitoring of their changes during fermentation

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ABSTRACT

In this work, an *ad hoc* method to identify and quantify polyphenols from caper berries was developed on high-performance liquid chromatography/electrospray ionisation source/mass spectrometry (HPLC–ESI–MS). The method was applied during fermentation carried out with *Lactobacillus pentosus* OM13 (Trial S) and without starter (Trial C). A total of five polyphenols were identified. All samples contained high concentrations of rutin. Epicatechin was found in untreated fruits, on the contrary quercetin was detected during fermentation. Trial S was characterised by a more rapid acidification and lower levels of spoilage microorganisms than Trial C. *L. pentosus* dominated among the microbial community of both trials and the highest biodiversity, in terms of strains, was displayed by Trial C. *Aureobasidium pullulans* was the only yeast species found. The analytical method proposed allowed a high polyphenolic compound recovery from untreated and processed caper berries in short time. The starter culture reduced the bitter taste of the final product.

1. Introduction

Capparis species belong to the *Brassicaceae* family, which included several vegetables, such as cabbage, brussels sprouts, cauliflower, red chicory, rocket, radish, etc. *Capparis spinosa* L. is largely cultivated in the Mediterranean basin for its flower buds (capers) and fruits (caper berries) that are consumed, after processing, for their flavour and digestive properties (Pérez Pulido et al., 2005). As fresh vegetables, caper berries are subjected to a rapid deterioration. Although recent techniques of caper transformation have been applied at industrial level, caper productions are still artisanal (Palomino et al., 2015). Capers are traditionally fermented in brine through spontaneous fermentation that is driven by lactic acid bacteria (LAB) (Pérez Pulido et al., 2005).

LAB are a major part of the fermenting microbiota of fermented plant foods (Daeschel, Andersson, & Fleming, 1987; Di Cagno, Coda, De Angelis, & Gobbetti, 2013). The species *Lactobacillus plantarum* and *Lactobacillus pentosus* are the starter LAB most widely used for table olive productions (Aponte et al., 2012; Hurtado, Reguant, Bordons, & Rozès, 2012). The unprocessed olive drupes cannot be eaten due to the presence of oleuropein that is a bitter glucoside compound (Hurtado et al., 2012) and, even though a variety of technological methods are applied to produce table olives, LAB fermentation is largely used to carry out the biological debittering process of this product (Marsilio, Lanza, & Pozzi, 1996). Similarly to olives, unprocessed caper products (flowers and berries) are characterised by a significant bitter taste due to presence of polyphenol compounds. Lactobacilli showing the capacity to metabolise phenolic compounds are usually isolated from fermented foods obtained from raw materials with high content of polyphenolic compounds (Svensson, Sekwati-Monang, Lutz, Schieber, & Gänzle, 2010).

Polyphenols are secondary metabolites that can be commonly found in many plants. Currently, they are receiving considerable attention for their health benefits such as cancer prevention, inflammatory disorders and cardiovascular diseases (Cicerale, Lucas, & Keast, 2010). All these positive effects appear to be strongly related to an anti-oxidative action that is primarily due to low-molecular-weight polyphenols (Dekanski et al., 2011).

Thus, the nutritional value and health benefits of the foods depend also on the type and amount of natural polyphenolic compounds.

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To our knowledge, no paper has been published on the determination of polyphenolic compounds from caper berries. Furthermore, scientific literature on caper flower polyphenols is still very limited. Within the class of flavonoids, only quercetin and rutin have been found. Furthermore, so far, only the species *L. plantarum* has been object of selection and application as starter in caper berry production (Palomino et al., 2015).

The aims of the present research were: to develop a analytical method for the identification and quantification of polyphenols of caper berry; to monitor their changes during caper berry transformation; to evaluate the performances of the strain *L. pentosus* OM13, well adapted to saline and phenolic matrices, to produce high quality fermented caper berries. Chemical, microbiological and sensory parameters were monitored during processing.

2. Materials and methods

2.1. Experimental caper berry production and sample collection

Caper berries were manually harvested from spontaneous fields within Trapani province (Sicily, southern Italy). The production process was performed in controlled conditions at the Agricultural Microbiology laboratory – food section – University of Palermo (Department of Agricultural and Forest Science).

The samples of caper berries were transferred into two vats (2.5-L volume). Each vat contained about 1.2 kg of caper berries and was filled in with a brine composed of NaCl 9% (w/v). One vat was inoculated with the autochthonous strain *L. pentosus* OM13 at approximately 7 Log CFU/mL (Trial S). The LAB starter OM13, proven to be highly adapted to saline conditions in presence of phenolic compounds, was isolated from olive drupes (Aponte et al., 2012) and is now used to produce fermented table olives at industrial level (Martorana et al., 2015, in press). The other vat was added with the same brine, but not inoculated with any starter culture, and spontaneously fermented as control production (Trial C). The experimentation was performed in triplicate (three vessels per trial). The fermentation of all trails was carried out at room temperature for 45 d and it was periodically monitored. Samples of brines (about 50 mL) were collected before starter inoculation, immediately after its addition (0) and at 3, 6, 9, 12, 15, 21, 33 and 45 d of fermentation.

2.2. Analysis of polyphenols

The analysis of polyphenols from caper berry samples was carried out by high-performance liquid chromatography/electrospray ionisation source/mass spectrometry (HPLC–ESI–MS). The extraction method (Becerra-Herrera, Sánchez-Astudillo, Rafael Beltrán, & Sayago, 2014; Talhaoui et al., 2014) was performed by using only methanol. The analysis of polyphenols from caper berry samples was carried out by high-performance liquid chromatography/electrospray ionisation source/mass spectrometry (HPLC–ESI–MS) on methanolic extracts by modifying literature methods (Becerra-Herrera et al., 2014; Talhaoui et al., 2014). An external calibration of commercial standards of polyphenol compounds was performed to determine the polyphenol concentrations in samples.

2.2.1. Standard solution

A standard stock solution containing rutin hydrate, gallic acid, 3,4-dihydroxycinnamic acid (caffeic acid), (+)-catechin-hydrate, *p*-coumaric acid, (−)-epicatechin, myricetin, quercetin and vanillic acid (Sigma–Aldrich, Milan, Italy) was prepared by adding weighed amount of each standard in methanol (HPLC grade) at concentration of 100 mg/L stored at 4 °C in the dark until use.

2.2.2. Extraction procedure

Aliquot of four grams of air-dried caper berry samples were added with methanol (10 mL) and homogenised with an Ultra-Turrax system (T 25 basic IKA labortechnik, Staufen Germany). The samples were left under stirring overnight. The mixture was centrifuged (4000 rpm × 15 min) and the supernatant was filtered through a 0.45 μm filter (Sartorius, Muggiò, Italy). The extraction was repeated twice. The two extracts were diluted in methanol and then gently dried. The residue was dissolved with methanol (200 μL) and then injected in HPLC. An external calibration was obtained by analysing nine standard solutions at different concentrations. Analyses were performed in triplicate to ensure stability and reproducibility of the method.

2.2.3. Chromatographic procedure and measurement

The chromatographic system consisted of Agilent 6130 Series Quadrupole LC/MS Systems with a G1311A Quaternary Pump, G1329A High Performance Autosampler, G1316A Thermostated Column Compartment and G1315D Diode Array Detector (DAD). Separation was carried out using an Agilent Eclipse XBD-C18 (4.6 × 150 mm, 5 μm) column. Identification and quantification of polyphenolic compounds were obtained using a G6120B Single Quadrupole LC/MS system equipped with an electrospray ionisation source (ESI). MS tune was optimised to the best experimental conditions. For target compound analysis, a flow injection analysis (FIA) was carried out to determine the fragmentor setting to improve the compounds responses. The potential chosen was 200 V. ESI work conditions were: capillary voltage 5000 V, gas flow rate 13 L/m, gas temperature 300 °C and nebuliser pressure 60 Psi. To obtain the best sensitivity, the quadrupole was used in SIM mode. Optimum separation was achieved with a binary mobile phase gradient at a flow rate 0.5 mL/m, the column temperature was kept at 30 °C and the injection volume was 5 μL. Solvents were (A) water/formic acid pH 3.1, and (B) acetonitrile. The gradient elution program was as follows: 0–15 min, 10–60% B, 15–20 min, 60–10% B.

2.3. pH determination and microbiological analyses

The pH value of samples was determined by a pH meter BASIC 20+(Crison Instrument S.A., Barcelona, Spain). Decimal dilutions of brines were prepared in Ringer's solution (Sigma–Aldrich). Different microbial groups were enumerated as follows: LAB on de Man-Rogosa-Sharpe (MRS) agar, incubated anaerobically at 30 °C for 48 h; total yeasts (TY) and filamentous fungi on dichloran rose bengal chloramphenicol (DRBC) agar, incubated aerobically at 25 °C for 5 d; *Enterobacteriaceae* on double-layer violet red bile glucose agar (VRBGA), incubated aerobically at 37 °C for 24 h; pseudomonads on *Pseudomonas* agar base (PAB) supplemented with CFC supplement, incubated aerobically at 20 °C for 48 h; staphylococci on Baird Parker agar (BPA) and coagulase positive staphylococci (CPS) on BPA added with RPF supplement, incubated aerobically at 37 °C for 48 h. Analyses were performed in triplicate. All media and the supplements used were supplied from Oxoid (Thermofisher, Basingstoke, UK).

2.4. Isolation, phenotypic and genotypic investigation of LAB

Presumptive LAB (at least 5 colonies with the same colour, morphology, edge, surface and elevation) were collected from the highest plated dilutions following their growth on MRS agar. The isolates were purified by successive sub-culturing and the purity of the isolates was checked microscopically. Gram-positive (Gregersen KOH method) and catalase negative (determined in presence of H_2O_2 5%, v/v) were stored in broth containing 20% (v/v) glycerol at −80 °C until further experimentations.

LAB were initially subjected to a phenotypic grouping based on cell morphology and disposition, determined by an optical microscope, growth at 15 and 45 °C and metabolism type, testing the produce of CO_2 from glucose. The last assay was carried out with the same growth media used for isolation, without citrate from which certain LAB can result in gas formation. The obligate homofermentative metabolism was determined by the absence of growth in presence of a mixture of pentose carbohydrates (xylose, arabinose, and ribose; 8 g/L each) in place of glucose.

DNA from LAB isolates was extracted using the InstaGene Matrix kit (Bio-Rad Laboratories, Hercules, CA) according to the manufacturer's instructions. Strain differentiation was performed by random amplification of polymorphic DNA-PCR (RAPD-PCR) analysis in a 25-μL reaction mix using single primers M13, AB111 and AB106 as previously described by Settanni et al. (2012). Due to the presumptive dominance of bacteria belonging to the *L. plantarum* group, the identification of the different strains at species level was performed by the multiplex PCR analysis based on the *rec*A gene with species-specific primers for *L. pentosus*, *L. plantarum* and *L. paraplantarum*, as described by Torriani, Felis, and Dellaglio (2001).

One representative culture for each multiplex cluster and all strains that did not show amplification with the multiplex PCR analysis were analysed by 16S rRNA gene sequencing as described by Weisburg, Barns, Pelletier, and Lane (1991). DNA sequencing reactions were performed at PrimmBiotech S.r.l. (Milan, Italy). The identities of the sequences were determined by BlastN search against the NCBI non-redundant sequence database located at http://www.ncbi.nlm.nih.gov and those available at EZTaxon located at http://www.ezbiocloud.net/eztaxon.

2.5. Isolation and genotypic identification of yeasts

Yeasts were collected from DRBC medium. At least five colonies per morphology were randomly collected from the agar plates, purified to homogeneity after several sub-culturing steps onto DRBC medium and subjected to genetic characterisation.

DNA extraction was performed as reported above. All selected isolates were preliminary grouped by restriction fragment length polymorphism (RFLP) analysis of the region spanning the internal transcribed spacers (ITS1 and ITS2) and the 5.8S rRNA gene as reported by Esteve-Zarzoso, Belloch, Uruburu, and Querol (1999). One isolate per group was identified at species level by sequencing the D1/D2 region of the 26S rRNA gene to confirm the preliminary identification obtained by RFLP analysis. D1/D2 region was amplified and polymerase chain reaction (PCR) products were visualised as described by Settanni, Sannino, Francesca, Guarcello, and Moschetti (2012). Reaction sequencing and species allotment were performed as reported above.

2.6. Sensory evaluation

The evaluation of the sensory profiles of the fermented caper berries was performed by using a descriptive method (ISO 13299:2003) as reported by Palomino et al. (2015). The sensory analyses were carried out after 15 and 45 d.

Twelve judges (6 females and 6 males, 22–48 years old) were trained in preliminary sessions using different samples of commercial caper berries in order to develop a common vocabulary for the description of the sensory attributes of the experimental samples, as well as to familiarise them with scales and procedures. Each attribute was extensively described and explained to avoid any doubt about the relevant meaning. A total of 7 descriptors were included in the analysis: hardness, fibrousness and crispness (rheological characteristics), acid, bitter and salty (taste) and negative sensations. The samples were randomly evaluated by assigning a score between 1.00 (absence of sensation) and 9.00 (extremely intense) in individual booths under incandescent white light.

2.7. Statistical and explorative multivariate analysis

Data of pH, microbiological investigation and sensory evaluation were analysed using a generalised linear model (GLM) that included the effects of samples; the Student "*t*" test was used for mean comparison. The post hoc Tukey's method was applied for pairwise comparison. Statistical significance was attributed to p values of <0.05. The resulting scores from the sensory analysis were averaged and compared. In order to analyse the effects both of judges and sensory descriptors the two way analysis of variance (ANOVA) was applied to identify significant differences among caper berry attributes.

In addition, an explorative multivariate analysis was employed to investigate relationship among data obtained from the different experimentations. A hierarchical cluster analysis (HCA) (joining, tree clustering) was carried out for grouping the trials according to their similarity, measured by Euclidean distances, whereas cluster aggregation was based on the single linkage method (Todeschini, 1998). Furthermore, the principal component analysis (PCA) was employed to investigate relationships among samples. The input matrix used for HCA and PCA consisted of the total area under growth/decline curves of LAB, yeasts, enterobacteria, pseudomonads, staphylococci, CPS as well as pH values (Bautista-Gallego et al., 2011; Blana, Grounta, Tassou, Nychas, & Panagou, 2014). Areas were calculated by integration using the OriginPro 7.5 sofware (OriginLab Corporation, Northampton, USA). In addition, other relevant indexes of pH and microbial changes were taken into account as follows: maximum and minimum values of pH, maximum and minimum values of microbial populations (Bautista-Gallego et al., 2011).

The number of principal factors was selected according to the Kaiser criterion (Jolliffe, 1986) and only factors with eigen-values higher than 1.00 were retained. All data were preliminary evaluated by using the Barlett's sphericity test (Mazzei, Francesca, Moschetti, & Piccolo, 2010) in order to check the statistically significant difference among samples within each data set.

Statistical data processing and graphic construction were achieved by using STATISTICA software version 10 (StatSoft Inc., Tulsa, OK, USA) and XLStat software version 7.5.2 (Addinsoft, New York, USA) for excel.

3. Results and discussion

3.1. Optimisation of analytical parameters

Samples of caper berries were analysed for polyphenols content by an analytical strategy developed in this study adapting and combining several extraction, separation and quantification techniques reported for other food matrices (Ryan & Robards, 1998), since there is no method available for the determination of polyphenols of this vegetable matrix.

The optimal experimental MS parameters were obtained by flow injection analysis (FIA). FIA was performed by injection of multiple samples within the same run without the column. The MS parameters subjected to optimisation were drying gas flow and temperature, nebuliser pressure and fragmentor voltage. FIA was also used to check quickly compound sensitivity or linearity. The individual polyphenolic compounds were identified and quantified by HPLC–ESI–MS. The compounds were identified by comparing their mass spectra determined from standard solutions.

In order to avoid or minimise interferences from background and side products, as well as to enhance the sensitivity, quantifica-

tion was performed using HPLC-MS in SIM mode. With exception of pseudo-molecular (protonated) ion of the derivative $[M+H]^+$, which was associated to the most abundant peak (Table 1), other characteristic peaks were found. For all compounds, a peak corresponding to M+1 was found which was attributed to the formation of $[M+H]^+$. The loss of OH $[M-17]^+$ were also detected. $[M+H]^+$ peak of each analyte was primarily exploited for quantitative purposes. Apart from $[M+H]^+$, the remaining most abundant ion of each species was used for confirmation purposes. The chromatographic conditions were optimised to obtain the best peak shape and analysis time. Analytical parameters of the method were evaluated in order to establish the method performance, ensuring the adequate identification, confirmation and quantification of the standard compounds. Linearity, trueness (expressed as recovery), limit of detection (LOD) and limit of quantification (LOQ) parameters were studied.

Method accuracy, expressed in terms of relative standard deviation (%RSD), was evaluated for each polyphenolic compound in standard solution at concentrations level of 5 μg/g. The %RSD values for the repeatability for each compounds were lower than 5%. Trueness was estimated through recovery studies, as follows: before extraction, different aliquots of sample were spiked at three levels, 10, 5, 2.5, μg/g with the standard solution (S_1) and they were extracted with the developed method. On the other hand, other aliquots of the same sample were extracted without spiking (S_0) and recovery was calculated as follows: $R = 100^*(S_1 - S_0)/C_{spiked}$. The linearity of the method was studied by the injection of standard solution at ten concentration levels. All calibration curves, which were obtained as a function of the integrated peak area, were linear over the studied range, with determination coefficients (r^2) > 0.99 for all components. The limit of detection (LOD) was determined as signal-to-noise ratio of 3:1 and the limits of quantification (LOQ) were determined as the signal-to-noise ratio of 10:1 and were calculated using the following equation:

$$LOD = 3 * S_b/m$$

$$LOQ = 10 * S_b/m$$

where S_b corresponds to the standard deviation of ten blank samples matrix and m stands for the slope of the calibration curve. Standard deviation was performed to assess the repeatability of the method.

For the quantification of the polyphenolic compounds, it must be taken into account that food samples are complex matrices. Thus, a large amount of compounds can interfere in the analyte signal, providing matrix effect (Document SANCO/12571/, 2013). Consequently, this effect was studied to ensure analytical results. Caper berries were spiked before the extraction, with the standard solution at different concentrations (2.5–5–10 μg/g) and the slopes of the calibration curves obtained were compared with the external calibration curves. A tolerable signal suppression or enhancement effect must be considered if the slope ratio (matrix/solvent) ranged between 0.8 and 1.2, whereas lower values than 0.8 or higher than 1.2 imply a strong effect in signal suppression or enhancement, respectively. With regards to all phenolic compounds, the results were included in the slope ratio. Thus, no matrix effect was observed. The different parameters of the method of extraction are summarised in Table 2.

Table 1
Retention time and mass spectrometry parameters of polyphenolic compounds.

Compound	Precursor ion (*m/z*)	Product ion	Retention time (*min*)
Gallic acid	170	153	4.2
Catechin	290	139	7.8
Epicatechin	290	139	8.6
Caffeic acid	179	135	8.8
Vanillic acid	168	151	9.1
Rutin	610	611	9.4
Coumaric acid	164	147	10.6
Myricetin	318	357	12.1
Quercetin	302	303	14.0

Symbols: *m*, mass; *z*, charge.

3.2. Analysis of polyphenols in caper berries

Polyphenols are the biggest nutrients group of phytochemicals. So far, no work has been focused on the polyphenols content of caper berries, even though caper fruits and other members of *Brassicaceae* family are object of investigation (Conforti et al., 2011; Kaulmann, Jonville, Schneider, Hoffmann, & Bohn, 2014). In particular, Kaulmann et al. (2014) reported the polyphenolic profiles of 27 samples of *Brassica oleracea* varieties and eleven polyphenols were identified. In this study, nine polyphenolic compounds that have been detected or searched in similar matrices, such as caper flowers (Conforti et al., 2011), other edible plants and berries (Gull, Anwar, Sultana, Cervantes Alcayde, & Nouman, 2015; Miean & Mohamed, 2001; Häkkinen, Kärenlampi, Heinonen, Mykkänen, & Törrönen, 1999) and processed vegetables (Aron & Kennedy, 2008), were investigated, but only five compounds were identified and analysed for their concentration in our samples (Table 2).

The extraction method described in this study allowed high polyphenolic compound recovery (typically higher than 85%) in short time. This method was combined with a powerful analytical technique (HPLC-MS) that determined a correct identification and reproducible quantification in a wide range of concentrations of phenolic compounds. An assessment of matrix effect has been detected, but it was negligible for all the phenolic compounds investigated. The result revealed the presence of phenolic acids (vanillic acid) and flavonoids (quercetin, rutin, (−)-epicatechin and myricetin). A total of five polyphenols compounds were identified on the unprocessed berries, in brine at the 15th d and at the end of the fermentation for both trails. Epicatechin was found only in untreated fruits, vanillic acid was identified in all samples below LOQ, myricetin was quantified at 5.2% in untreated berries, but below LOQ in the other samples. All samples contained high concentrations of rutin (from 63% to 100%), while quercetin was identify in Trial C only at the 48th d (37%), while in Trial S at the 15th d (7.7%) and at the 45th d (19%).

Among the phenolic compounds detected in this study, quercetin, and rutin were also found in caper fruits (Conforti et al., 2011; Khalidi, Triki, & Munne-Bosch, 2010). However, the presence of rutin, the major phenolic compound of caper berries as revealed by our study, is reported at lower concentrations for caper flowers (Khalidi et al., 2010).

Results from this study indicate that fermented caper berries are characterised by a polyphenolic profile different from that of the untreated fruits. Since quercetin was not found in fresh caper berries, we assume that this compound compared during fermentation as hydrolysis by-product of rutin. This because rutin can be hydrolysed by a-rhamnosidase to produce quercetin-3-glucoside, and then further hydrolysed by β-glucosidase to produce quercetin (Lin et al., 2014). However, rutin can be also hydrolysed directly by hesperidinase to produce quercetin (Tranchimand, Brouant, & Iacazio, 2010).

3.3. pH values and microbiological loads

Physico-chemical and microbiological characteristics of brine samples collected during the manufacturing process are reported in Fig. 1. The values of pH were 4.3 in caper berries and 7.3 in brine on average before the beginning of transformation. During fermen-

Table 2
Analytical parameters for the polyphenols extraction and concentration of phenolic compounds (μg/g) in caper berries.

Compound	Recovery (%)	LOD	LOQ	r^2	Caper berries				
					Untreated	15 d of fermentation		45 d of fermentation	
						Trial S	Trial C	Trial S	Trial C
Epicatechin	98.4	0.016	0.04	0.9971	1.04 ± 0.06	n.d	n.d	n.d	n.d
Vanillic acid	97.1	0.017	0.04	0.9982	<LOQ	<LOD	<LOQ	<LOQ	<LOQ
Rutin	102.3	0.014	0.03	0.9983	21.08 ± 0.26	61.27 ± 4.18	23.35 ± 0.25	33.27 ± 2.30	1.60 ± 0.11
Myricetin	97.6	0.014	0.04	0.9945	1.20 ± 0.11	<LOD	<LOQ	<LOQ	<LOQ
Quercetin	104.8	0.027	0.05	0.9981	n.d	5.15 ± 0.68	n.d	7.82 ± 0.23	0.93 ± 0.01

Abbreviations: LOD, limit of detection; LOQ, limit of quantification; n.d., not determined; *Symbols*: r^2, determination coefficients.

tation, pH ranged between 7.1 (d 0) and 3.5 (d 45) for both trials. Trial S showed the highest decrease of pH during the first days of fermentation, but at the 45th d of observation no difference of pH value was found between the trials. The microbiological characteristics of untreated capers were as follows: 2.81 CFU/mL of LAB, 3.21 Log CFU/mL of yeasts, 3.57 Log CFU/mL of *Enterobacteriaceae*, 2.28 Log CFU/mL of pseudomonads and 3.33 Log CFU/mL of staphylococci.

Trial S was characterised by a LAB concentration of 7.56 Log CFU/mL immediately after inoculation. From the 3rd d of fermentation, when LAB were found at 7.45 Log CFU/mL, their concentration decreased over time reaching the lowest value (3.06 Log CFU/mL) at 33 d. The presence of LAB in Trial C was at lower loads than Trial S for the entire period of monitoring; the highest value of LAB was registered at 9 d with 3.78 Log CFU/mL. LAB were no more detected at the 45th d in both trials. Yeasts were counted until the 3rd d (3.00 Log CFU/mL for Trial S and 3.79 Log CFU/mL for Trial C).

After inoculation with starter culture, the *Enterobacteriaceae* population decreased more rapidly in Trial S, were they were undetectable at the 3rd d, than Trial C which hosted 2.43 Log CFU/mL of enterobacteria at the same time of collection. However, *Enterobacteriaceae* disappeared from the 6th d also in Trail C. Staphylococci and pseudomonads were undetectable, in both trials, even during the first days of analysis.

To our knowledge, very limited information are available on the microbiological characteristics of caper berries. Pérez Pulido et al. (2005) focused on the dynamics of the LAB community during spontaneous fermentation of caper berries. Recently, Palomino et al. (2015) monitored the microbial evolution of caper fruits inoculated with selected *L. plantarum* as starter cultures. In both works (Palomino et al., 2015; Pérez Pulido et al., 2005) the authors followed the fermentation process up to 7 d. However, Özcan (1999) stated that a longer monitoring of the microbial populations is recommended. In order to better evaluate the microbial development and the interactions among different populations and to investigate their effects on the evolution of the polyphenolic profiles, the caper berry transformation was followed until 45 d both for a spontaneous fermentation and for a driven process. The behaviour of LAB and *Enterobacteriaceae*, as well as pH kinetics, registered in our work confirmed the observation of Pérez Pulido et al. (2005) and Palomino et al. (2015). However, an opposite trend was displayed by yeasts, which in our work were detected at the beginning of the process, while in the work of Pérez Pulido et al. (2005) at the end of fermentation.

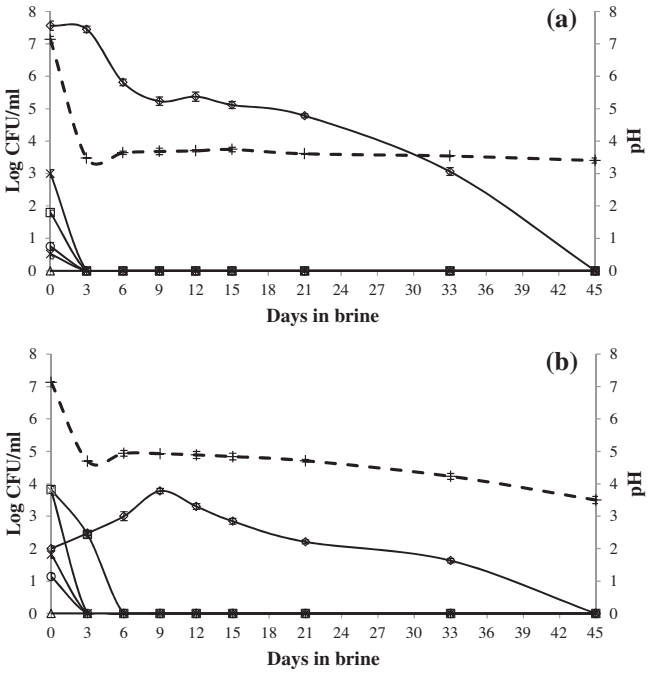

Fig. 1. Changes in pH values and microbial counts during caper berry preparation. Changes in pH and microbial counts of caper berry brines added with *Lactobacillus pentosus* OM13 (a) and spontaneously fermented (b). pH (|), lactic acid bacteria (◇), *Enterobacteriaceae* (□), total aerobic mesophilic bacteria (△), total yeasts (×), staphylococci (∗), pseudomonads (○).

3.4. Isolation, strain typing, identification and distribution of LAB species

A total of 971 colonies were collected from the highest plated dilutions of cell suspensions on MRS medium. After microscopic inspection, Gram and catalase tests, 742 rods were still considered presumptive LAB cultures, as being Gram-positive and catalase negative. The preliminary phenotypic characterisation did not allow the separation of the isolates into different groups, since all cultures were able to grow at 15 °C, unable to develop at 45 °C and were facultative homofermentative (grew in presence of pentose carbohydrates, but CO_2 was not produced from glucose).

Due to the high number of isolates, about 40% of cultures representative of the different samples (experimental trials and time of collection) was subjected to RAPD analysis which allowed the identification of 27 different strains (data not shown).

Multiplex PCR analysis of the *rec*A gene revealed the presence of a major group of *L. pentosus* composed of 23 strains (data not shown). Analysis of 16S rRNA gene confirmed that the strains showing amplification products with the multiplex PCR were allotted into the species *L. pentosus* (Acc. No. KT724952). This species dominated the LAB population of both trials. The rest of the strains, isolated only from berries of Trial C at the 9th and 12th d, were identified as *L. plantarum* (Acc. No. KP987526). Trial C showed a high biodiversity, in terms of strain number, within the species *L. pentosus*. As expected, the commercial starter OM13 was the strain most frequently isolated during the entire transformation process of caper berries in Trial S.

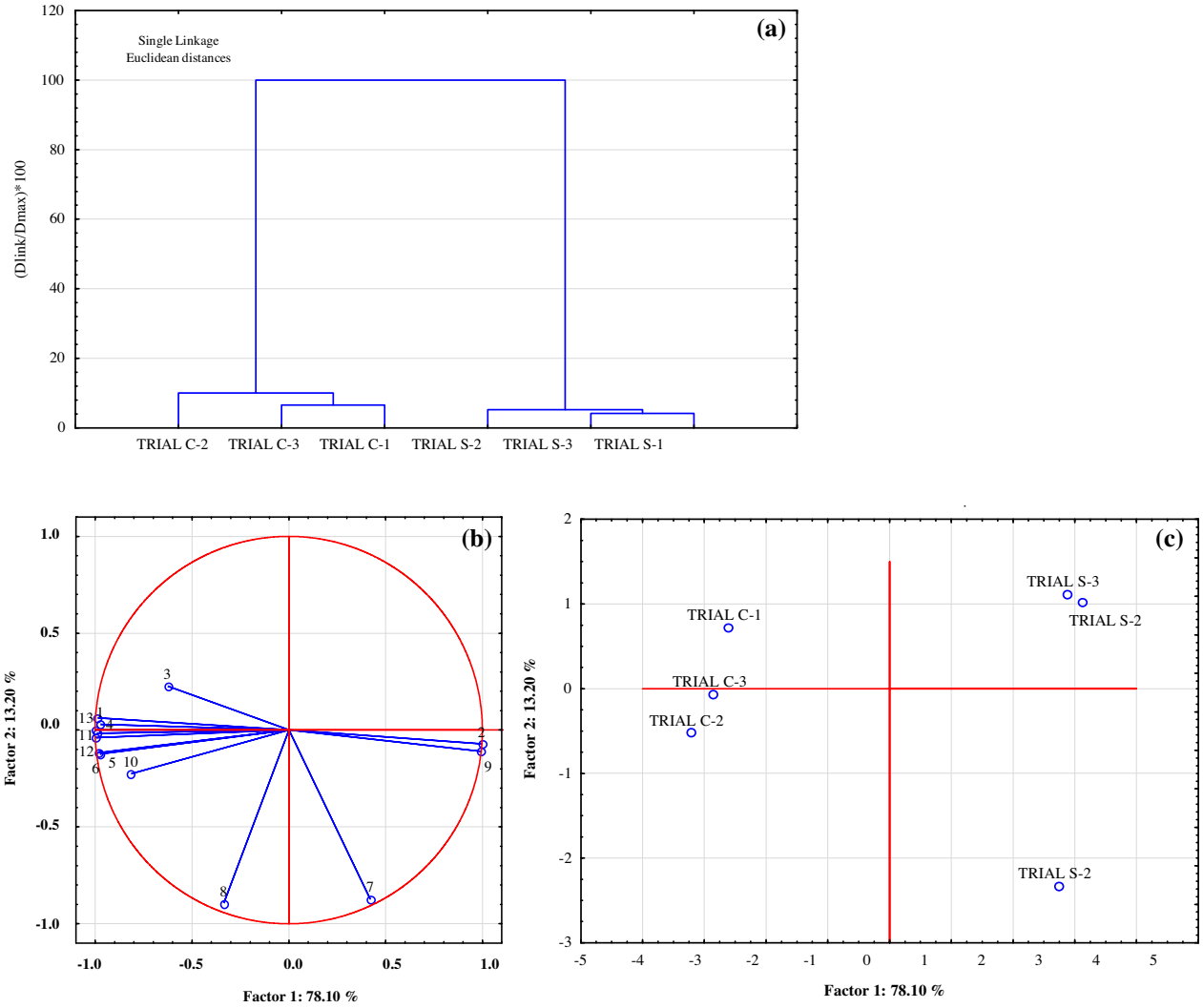

Fig. 2. Dendrogram resulting from HCA analysis (a), loading plot (b) and score plot (c) of PCA analysis based on values of pH and microbial changes of brine samples during the production of caper berries. *Abbreviations:* TRIAL S, experimentation inoculated with starter *Lactobacillus pentosus* OM13; TRIAL C, experimentation spontaneously fermented. Numbers associated to trial codes correspond to replicates per each trial; Numbers associated to loading plot (b): 1, MinpH; 2, MaxpH; 3, MaxMRS; 4, MRS; 5, MaxDRBC; 6, DRBC; 7, VRBGA; 8, MaxBPA; 9, MaxVRBGA; 10, BPA; 11, PAB; 12, pH; 13, MaxPAB.

Even though the trial C was not inoculated with starter, the species *L. pentosus* dominated the LAB population. These results are in contrast with those reported for the same matrix by other authors (Palomino et al., 2015; Pérez Pulido et al., 2005) who found *L. plantarum* as the main fermenting agent. A spontaneous fermentation might be highly variable, as function of the microbial diversity of the raw material, and the quality of the final product is consequently unpredictable. A starter culture is a microbial culture containing a large number of viable microorganisms, belonging to single or multiple species, added to begin the fermentation process (Holzapfel, 2002). The use of a starter culture reduce the risk of spoilage and variability of the final products. LAB are generally employed as starter also because they contribute significantly to the flavour, texture, nutritional value and microbial safety of the fermented foods (Settanni & Corsetti, 2008). In addition, the starters with probiotic properties confer also functional properties to foods (Silvestri, Francesca, Settanni, & Moschetti, 2009).

3.5. Isolation, identification and distribution of yeasts

One hundred and seventy-eight yeast colonies were collected from DRBC agar and subjected to the molecular identification. All isolates showed identical length (about 590 bp) of 5.8S-ITS amplicons. After the restriction analysis of this genomic region, the isolates were clustered into a single group characterised by the RFLP profile of *Aureobasidium pullulans* (Sinacori et al., 2014). The sequencing of D1/D2 domain of the 26S rRNA gene confirmed the identification of the yeasts as members of *A. pullulans* species (Acc. No. KP987525). It was found only at the beginning of the transformation process in both trials. The species *A. pullulans* has been detected in other food environment, mainly during wine production (Francesca et al., 2014). This is the first work reporting the isolation of yeasts from caper berries. However, due to their disappearance after the beginning of fermentation, probably *A. pullulans* yeasts do not play any role during fermented caper berry production.

3.6. Sensory analysis

The sensory analysis was performed at 15 and 45 d. The data of the last evaluation are reported in Table S1. Caper berries obtained with *L. pentosus* OM13 added as starter strain differed significantly ($p < 0.05$) from those transformed through spontaneous fermentation. The main differences were estimated in terms of hardness and

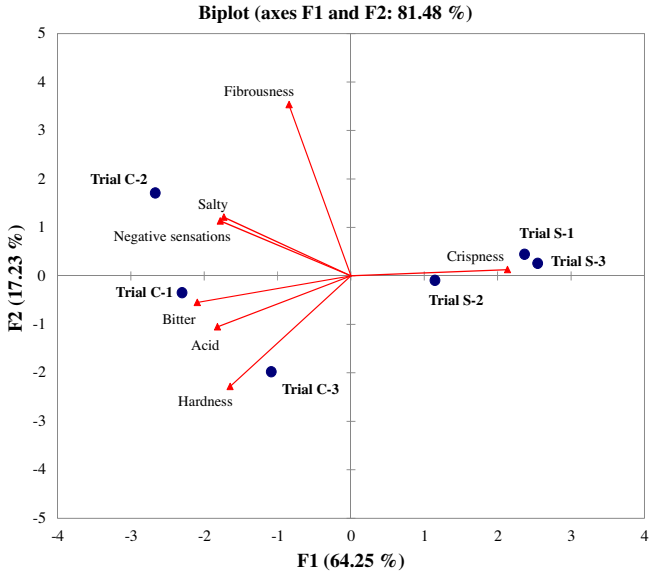

Fig. 3. PCA for sensory data of caper berries at the end of process (d 45). Biplot graph show relationships among factors, variables and trials. *Abbreviations:* Trial S, experimentation inoculated with starter *Lactobacillus pentosus* OM13; Trial C, experimentation spontaneously fermented; the numbers associated to trial codes correspond to replicates per each trial.

bitterness. In details, the fermented fruits from Trial S were characterised by a high hardness and low bitter taste. However, all samples showed very low values of negative sensations.

3.7. Multivariate statistical analysis

HCA classified the trials in accordance to their mutual dissimilarity and relationship by using the 18 variables, including pH and microbial analysis results (Fig. 2a). Replicates of all trials were clearly separated into two mega-cluster (Trial S and Trial C) in accordance to the method of fermentation. Within the mega-cluster of Trial C, the replicate C-3 was found at a level of dissimilarity higher than that of the others.

The results of pH and microbial counts were also subjected to PCA (Fig. 2b,c). A total of two (accounting for 78.07% and 13.29% of total variability) eigen-values higher than 1 were found. These results indicated that the initial 18 variables (used as data matrix for PCA) might be expressed as linear combination of two Factors explaining 91.30% of total variance. The components of the PCA were correlated to variables as shown in Fig. 2b and the corresponding values of factor loadings are reported in the Table S2. The discrimination of trials can be visualised in the plot of the scores (Fig. 2c). In detail, Fig. 2c, shows the projection of the cases (representing the three replicates per each trials) onto the planes as function of the Factors 1 and 2. The trials were significantly separated along Factor 1 on the basis of the fermentation method. These results confirmed that obtained by HCA.

The multivariate statistical analysis was concluded on data of sensory analysis and the biplot graphical representation is reported in Fig. 3. The caper berries inoculated with the starter strain OM13 resulted clearly separated along the F1 component and closely associated only to crispness. On the other hand, all replicates of capers spontaneously fermented were mainly related with bitter and hardness.

The Barlett's sphericity test was applied to all data matrix inputs and differences statistically significant ($p < 0.0001$) were found among trials.

4. Conclusions

Our study reports the description of a rapid, efficient and sensitive method based on HPLC–ESI–MS for the identification and quantification of polyphenolic compounds from caper berries. This method allowed the correct identification and the reproducible quantification of these compounds in a wide range of concentrations. Our study represents the first report on the analysis of polyphenols from caper berries. The results showed for caper berries a number of polyphenolic compounds higher than those reported in literature for caper flowers. Furthermore, the concentrations of the compounds known to be present in caper flowers were found at higher levels in caper berries.

The effects of *L. pentosus* as culture starter to produce caper berries were evaluated. The addition of the strain OM13 ensured the rapid decrease of pH as well as the low growth of potential spoilage microorganisms. Furthermore, the sensory evaluation showed that the caper berries fermented with the starter culture were characterised by a less bitter taste than those subjected to the spontaneous fermentation.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.foodchem.2015.10.045.

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