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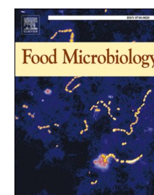

Study of the effects of pasteurization and selected microbial starters on functional traits of fermented table olives

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A R T I C L E I N F O

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A B S T R A C T

Table olives are one of the most known fruit consumed as fermented food, being a fundamental component of the Mediterranean diet. Their production and consumption continue to increase globally and represent an important economic source for the producing countries. One of the most stimulating challenges for the future is the modernization of olive fermentation process. Besides the demand for more reproducible and safer production methods that could be able to reduce product losses and potential risks, producers and consumers are increasingly attracted by the final product characteristics and properties on human health.

In this study, the contribution of microbial starters to table olives was fully described in terms of specific enzymatic and microbiological profiles, nutrient components, fermentation-derived compounds, and content of bioactive compounds. The use of microbial starters from different sources was tested considering their technological features and potential ability to improve the functional traits of fermented black table olives.

For each fermentation assay, the effects of controlled temperature (kept at 20 °C constantly) versus not controlled environmental conditions (oscillating between 7 and 17 °C), as well as the consequences of the pasteurization treatment were tested on the final products. Starter-driven fermentation strategies seemed to increase both total phenolic content and total antioxidant activity. Herein, among all the tested microbial starters, we provide data indicating that two bacterial strains (*Leuconostoc mesenteroides* KT 5-1 and *Lactiplantibacillus plantarum* BC T3-35), and two yeast strains (*Saccharomyces cerevisiae* 10A and *Debaryomyces hansenii* A15-44) were the better ones related to enzyme activities, total phenolic content and antioxidant activity. We also demonstrated that the fermentation of black table olives under not controlled environmental temperature conditions was more promising than the controlled level of 20 °C constantly in terms of technological and functional properties considered in this study. Moreover, we confirmed that the pasteurization process had a role in enhancing the levels of antioxidant compounds.

1. Introduction

Table olives are currently one of the most economically important fermented plant products in the food industry around the world (Caponio et al., 2019). The International Olive Council estimates that Spain, Greece, and Italy provide nearly 30% of the world's annual table olive production (IOC, 2018).

As widely known, the unprocessed olive drupes cannot be readily consumed, since they contain oleuropein and ligstroside, compounds that are responsible for the olives characteristic bitter taste (Hurtado et al., 2012). The three most important commercial preparations of table olives on the international market are Spanish green olives (treated green olives in brine), oxidized black olives (olives darkened by oxidation), and black olives in brine (natural black olives) (IOOC, 2004;

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Table 1
Yeast and bacterial strains used in this study.

Strain	Code	Origin
Staphylococcus pasteuri SB42	**B**	Miso
Leuconostoc mesenteroides KT 5–1	**C**	Table olives (Tufariello et al., 2015)
Lactiplantibacillus plantarum C 180–11	**D**	Table olives (Tufariello et al., 2015)
Lactiplantibacillus plantarum TB 11–32	**E**	Table olives (D'Antuono et al., 2018)
Lactiplantibacillus plantarum BC T3-35	**F**	Table olives (D'Antuono et al., 2018)
Candida parapsilosis CY 28	**1D**	Coffee
Saccharomyces cerevisiae 10A	**3D**	Table olives
Saccharomyces cerevisiae LI 180–7	**5D**	Table olives (Tufariello et al., 2015)
Saccharomyces cerevisiae KI 30–16	**7D**	Table olives (Tufariello et al., 2015)
Debaryomyces hansenii A 15–44	**8D**	Table olives (Tufariello et al., 2015)

Tufariello et al., 2016; Garrido-Fernández et al., 1997). In particular, natural black olives traditionally produced by a spontaneous fermentation process contain high levels of bioactive compounds (Romero et al., 2004; Tufariello et al., 2016) and are highly appreciated by the consumers for their content in aromatic compounds.

According to the natural black olives method, the fruits are placed directly in brine with a salt concentration ranging between 6 and 10% (w/v), thus permitting fermentation for at least 4–8 months directed by mixed populations of microorganisms, generally represented by the epiphytic microflora of yeasts and lactic acid bacteria (LAB) (Brenes et al., 2004; Romero et al., 2004; Hurtado et al., 2008).

Microbial starters, temperature and pasteurization conditions are the most important factors influencing production, quality and safety aspects of fermented table olives. Indeed, the use of starter cultures have been proposed as a new technological approach to control and standardize the fermentation process, improving the transformation step (Leal-Sánchez et al., 2003; Panagou et al., 2008; Marsilio et al., 2005; Servili et al., 2006; Sabatini and Marsilio, 2008), and including the use of selected Lactic Acid Bacteria (LAB) strains. Additionally, the role of yeasts in table olives production process and their potential applications as starters has recently been proposed, alone or in combination with LAB (Arroyo-López et al., 2012; Bevilacqua et al., 2013; Bonatsou et al., 2015; Chytiri et al., 2020; Heperkan, 2013; Tufariello et al., 2016). In particular, yeasts can participate in the production of volatile compounds and metabolites that could play several roles: improving organoleptic characteristics of the final product (Garrido Fernández et al., 1995); releasing nutritive compounds that promote LAB growth (Nisiotou et al., 2010; Bautista-Gallego et al., 2011); controlling the development of undesired microorganisms (Psani and Kotzekidou, 2006). However, the use of starter cultures remains limited among processors due to several reasons, such as: cost, complexity, limited availability of knowhow on the selection process, validation of industrial starters (Tufariello et al., 2015).

Certainly, the use of single and multifunctional starter cultures in table olive fermentation becomes attractive for the food industry as it contributes to the reduction of process cost time, and risk of spoilage. Moreover, it contributes in enhancing the sensory characteristics of the final product and ensuring product safety (Servili et al., 2006; Benítez-Cabello et al., 2019; Vaccalluzzo et al., 2020).

Together with the microorganisms, another key parameter in the table olives production is the temperature at which the fermentation process is run. In fact, it is a paramount factor as it can influence the numbers and the type of microorganisms, select the prevailing species, affect the development of end products, and have physicochemical impact on olives, as influencing the tissues permeability. The traditional production of table olives is run in processing plants, where fermenters are exposed to open air and fermentation temperature is fluctuating.

At the end of the production process, table olives can be preserved as bulk preparations through a balance of several parameters, such as: pH (<4.3), salt (6% w/v sodium chloride or more), and acid addition (i.e., lactic acid 0.3% w/v) (IOOC, 2004). Alternatively, the finished products can be stored in a fresh brine, or in a fermentation brine, or a combination of the two previous ones (Grounta et al., 2013; Medina et al., 2013).

Natural black olives are generally distributed and sold at retail either in pasteurized preparations or without pasteurization. In this case, the olives are packed in plastic containers just after being washed and fermentation brine being replaced by a fresh brine with controlled pH. Pasteurization is widely considered one of the most effective preservation methods for killing pathogens in food products, with minimal negative effects on food quality. This strategy is widely accepted as internationally recognized standard to conform any heat treatment process for unprocessed products by the European Parliament and the Council of the European Union regulation on the hygiene of foodstuffs (Commission Regulation, 2004). Recently published research works have reported the impact of thermal pasteurization on the quality of fruits and vegetables, including parameters such as color, texture, vitamins, carotenoids and phenolics content, antioxidant activity, and many other nutritional attributes (Brenes and Kailis, 2021; Dias-Martins et al., 2020; Jafarpour, 2022).

Table olives are attracting growing interest due to their reported health benefits, intrinsically linked to the high content of monounsaturated fatty acids, as well as their antioxidant capacity, due to minor compounds such as tocopherols and phenols (Malheiro et al., 2011; Boskou et al., 2015). Furthermore, natural black olives fermentation method allows to achieve a much higher content of polyphenols respect to other traditional processing methods.

Polyphenols content of table olives can be also influenced by a thermal processing. In this case, two different events can occur, the mobilization of bound phenolics from the plant structures, due to heat breakups of the cell walls, together with the concurrent oxidation or degradation of phenolics species, widely sensitive to heat exposure (Arfaoui, 2021).

In this work, in order to evaluate the effects on quality and nutritional traits of fermented black table olives (*Cellina di Nardò*), the combination of three different factors as: 1) selected microbial starters (Bleve et al., 2014; Tufariello et al., 2015), 2) constant and controlled temperature (20 °C) versus ambient not controlled temperature (NCT), 3) pasteurization of final product, was investigated.

A multiparametric approach was successfully applied to determine the most favorable conditions for table olives fermentation, processing, and stabilization. Therefore, the evidences presented in this study could be beneficial to the industry for the optimization and further control of fermentation processes, thus achieving an impactful improvement in the finish products.

2. Materials and methods

2.1. Olives fermentations

2.1.1. Olive samples and preparation

Olives (cv *Cellina di Nardò*), provided by the company Eurolive spa (Lecce, Italy), were picked in December 2020, at an advanced ripening stage. They were immediately brought to the laboratory to perform the laboratory-scale fermentation experiments in triplicate. Samples of healthy black olives (each one of about 1.0 kg) were prepared following the standard company's procedure. They were washed, selected for a 10–12 mm caliber, and placed in glass jars of 1.5 kg capacity filled with 500 mL of brine containing 10% (w/v) NaCl at pH 4.45, adjusted with citric acid.

Two different conditions were chosen to test the best strategy between i) the incubation at the environmental temperature (NCT), generally used by table olives industrial producers, to promote interactions among the microbial starters and the olive matrix, especially in terms of quality/safety traits and the nutritional features of the fermented table olives; ii) the maintenance of a constant incubation

Table 2
Microbiological assays on brines from fermented table olives samples at the end of the process (30 days). (A) controlled (20 °C) and at (B) uncontrolled environmental conditions (NCT). CNT: spontaneous fermentation, as control. BP: before pasteurization; AP: after pasteurization. Different letters in the same line indicate significant differences among samples ($p < 0.05$).

A											
	BACTERIA					YEASTS					
	Log_{10} CFU/ml (Mean ± SD)					Log_{10} CFU/ml (Mean ± SD)					
MICROORGANISMS	B	C	D	E	F	1D	3D	5D	7D	8D	CNT
TBC	9,85 ± 6,08[c]	5,88 ± 4,83[a]	7,15 ± 6,11[b]	6 ± 4,91[a]	6 ± 4,61[a]	6,78 ± 4,87[b]	6,88 ± 4,68[b]	4,90 ± 3,75[a]	7,72 ± 5,53[b]	6,65 ± 4,79[b]	5,51 ± 3,94[a]
LAB	8,36 ± 5,92[c]	6 ± 4,67[b]	5,51 ± 3,54[b]	6,46 ± 4,81[b]	7,59 ± 5,88[c]	6 ± 4,52[b]	7,08 ± 4,89[c]	6,95 ± 4,75[c]	6,83 ± 4,87[b]	7,63 ± 6,38[c]	3,90 ± 2,65[a]
Yeast and moulds	7,15 ± 5,65[c]	6,04 ± 4,64[b]	6,04 ± 4,52[b]	6,26 ± 4,33[b]	6,38 ± 4,52[b]	6,90 ± 4,83[c]	6,76 ± 4,74[b]	6,36 ± 4,63[b]	5,38 ± 3,58[a]	6 ± 4,36[b]	4,88 ± 3,30[a]
B											
	BACTERIA Log_{10} CFU/ml (Mean ± SD)					YEASTS Log_{10} CFU/ml (Mean ± SD)					
MICROORGANISMS	B	C	D	E	F	1D	3D	5D	7D	8D	CNT
TBC	4,40 ± 3,92[a]	4,18 ± 3,63[a]	4,48 ± 3,99[a]	4,18 ± 3,81[a]	4,65 ± 3,94[a]	5,85 ± 4,34[b]	5,18 ± 4,81[a]	4,95 ± 4,88[a]	5,18 ± 3,87[b]	6,65 ± 5,07[c]	4,51 ± 3,78[a]
LAB	4,26 ± 3,53[b]	5,70 ± 4,94[c]	5,18 ± 4,72[c]	3,90 ± 2,99[a]	4,59 ± 3,75[b]	5,18 ± 4,82[c]	5,08 ± 3,63[b]	4,85 ± 3,83[b]	4,83 ± 3,99[b]	5,63 ± 4,77[c]	3,10 ± 2,18[a]
Yeast and moulds	5,34 ± 4,04[a]	6,58 ± 5,88[b]	5,60 ± 4,87[a]	5,48 ± 4,74[a]	5,41 ± 4,53[a]	6,54 ± 5,75[b]	6,92 ± 5,88[b]	6,88 ± 5,94[b]	6,89 ± 5,65[b]	6,97 ± 5,93[b]	5,75 ± 3,87[a]

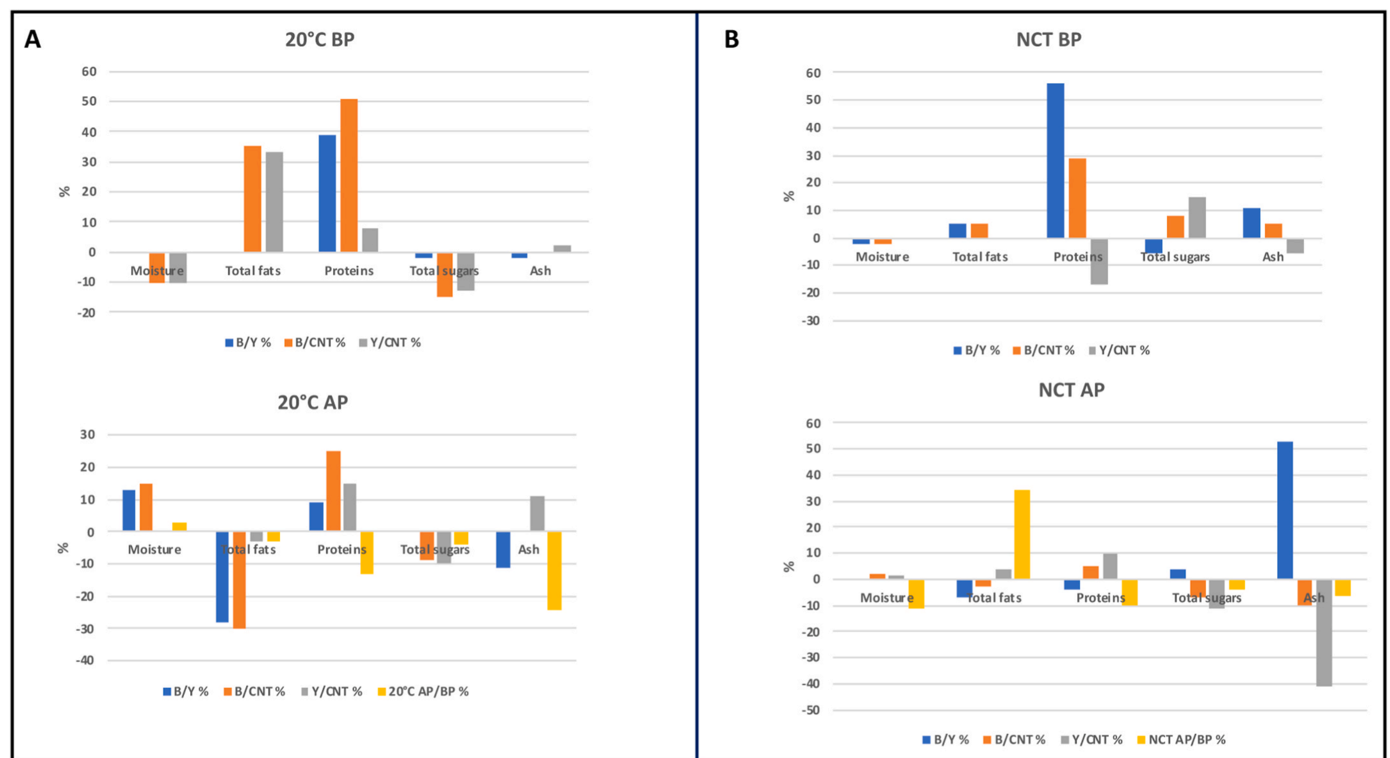

Fig. 1. Elaboration of the effects produced by the two different fermentation temperatures 20 °C (A) and not controlled temperature (B) and by the pasteurization step on the two sets of fermented samples on the chemical composition of table olives (data reported in Table S2). B: mean value % of bacterial inoculated table olives samples; Y: mean value % of yeast inoculated table olives samples; CNT: spontaneous fermentation; BP: mean value % of bacterial + yeast inoculated table olives samples before pasteurization; AP: mean value % of bacterial + yeast inoculated table olives samples after pasteurization.

temperature (20 °C) essentially favoring the mesophilic microbial growth.

Then, olives were allowed to ferment at: 1) a constant temperature of 20 °C in static conditions and 2) at environment temperature (during December 2020–January 2021), with a temperature ranging from 7 to 17 °C, both in the presence or absence of starter inocula.

2.1.2. *Microbial starter cultures preparation and fermentation conditions*

Bacterial and yeast starter cultures reported in Table 1 were used to drive olive lab-scale fermentations, by a single starting inoculum. Yeasts were grown in YPD medium (yeast extract 1% w/v, meat peptone 2% w/v, glucose 2% w/v, and agar 2% w/v) and incubated at 28 °C for 48 h. LAB was grown in Man, Rogosa, and Sharpe (MRS-glucose, LABM, UK) and *Staphylococcus pasteuri* Sb42 in MRS added with 2% (w/v) sucrose

Table 3
Organic acids in table olives fermented samples at the end of the process (30 days). Concentration values are expressed in (μg/mg DW). (A) controlled (20 °C) and (B) uncontrolled environmental conditions (NCT). CNT: spontaneous fermentation, as control.

A								
Sample	Glutamic acid	Malic acid	Shikimic acid	Lactic acid	Acetic acid	Pyruvic acid	Citric acid	Succinic acid
(m ± σ)								
20 °C BP								
B	20 ± 5	9 ± 2	0.38 ± 0.09	24 ± 6	10 ± 3	1.2 ± 0.3	34 ± 8	0.60 ± 0.15
C	75 ± 20	10 ± 3	0.60 ± 0.15	17 ± 4	5.0 ± 1.2	3.8 ± 0.9	51 ± 11	2.0 ± 0.5
D	49 ± 11	10 ± 3	0.55 ± 0.15	16 ± 4	6.1 ± 1.5	10 ± 3	45 ± 9	13 ± 3
E	55 ± 15	12 ± 3	0.45 ± 0.11	18 ± 5	4.9 ± 1.1	2.7 ± 0.7	65 ± 15	4.1 ± 1.0
F	30 ± 7	10 ± 3	0.50 ± 0.10	17 ± 4	2.7 ± 0.7	5.1 ± 0.8	49 ± 10	4.5 ± 1.1
1D	80 ± 20	11 ± 3	0.55 ± 0.15	15 ± 4	5.2 ± 0.9	8.6 ± 1.9	56 ± 14	5.5 ± 1.5
3D	48 ± 9	12 ± 3	0.50 ± 0.10	11 ± 3	4.8 ± 1.0	3.2 ± 0.8	55 ± 16	12 ± 3
5D	44 ± 10	7.0 ± 1.5	0.50 ± 0.15	13 ± 3	8 ± 2	3.1 ± 0.8	64 ± 13	6.2 ± 1.4
7D	500 ± 150	9 ± 2	0.81 ± 0.18	14 ± 3	7.9 ± 1.9	7.0 ± 0.14	85 ± 20	14 ± 3
8D	110 ± 30	11 ± 3	0.79 ± 0.17	11 ± 3	8.5 ± 1.8	5.5 ± 1.5	59 ± 11	9.1 ± 1.9
CNT	30 ± 70	16 ± 4	0.71 ± 0.15	10 ± 3	3.9 ± 1.0	8 ± 2	62 ± 15	14 ± 4
20 °C AP								
B	20 ± 5	9 ± 2	0.45 ± 0.10	25 ± 6	11 ± 3	3.4 ± 0.8	43 ± 10	11 ± 3
C	24 ± 6	13 ± 3	0.55 ± 0.15	14 ± 4	4.0 ± 1.0	3.3 ± 0.7	63 ± 12	14 ± 4
D	34 ± 8	15 ± 4	0.65 ± 0.15	15 ± 4	2.7 ± 0.6	4.1 ± 1.1	84 ± 20	14 ± 4
E	25 ± 6	6.0 ± 1.5	0.35 ± 0.09	10 ± 3	ND	ND	47 ± 13	ND
F	28 ± 7	8 ± 2	0.31 ± 0.08	15 ± 4	ND	2.9 ± 0.7	35 ± 9	14 ± 4
1D	20 ± 5	5.5 ± 1.5	0.26 ± 0.07	7.0 ± 1.5	ND	ND	31 ± 8	16 ± 4
3D	14 ± 3	14 ± 3	0.35 ± 0.09	5.5 ± 1.4	2.4 ± 0.6	3.0 ± 0.8	65 ± 15	14 ± 3
5D	13 ± 3	10 ± 3	0.37 ± 0.09	7.1 ± 1.6	5.2 ± 1.1	3.2 ± 0.8	62 ± 17	13 ± 3
7D	15 ± 4	10 ± 3	0.40 ± 0.10	5.4 ± 1.4	2.17 ± 0.15	2.0 ± 0.5	50 ± 10	1.9 ± 0.5
8D	14 ± 4	4.5 ± 1.0	0.35 ± 0.09	4.9 ± 1.0	5.7 ± 1.6	6.1 ± 1.5	41 ± 10	6.6 ± 1.5
CNT	13 ± 3	9 ± 2	0.38 ± 0.09	5.1 ± 1.1	1.3 ± 0.3	2.4 ± 0.6	38 ± 9	3.1 ± 0.8

B								
Sample	Glutamic acid	Malic acid	Shikimic acid	Lactic acid	Acetic acid	Pyruvic acid	Citric acid	Succinic acid
(m ± σ)								
NCT BP								
B	120 ± 30	13 ± 3	0.45 ± 0.10	13 ± 3	10 ± 3	3.3 ± 0.8	55 ± 15	28 ± 7
C	130 ± 30	6.7 ± 1.5	0.55 ± 0.15	12 ± 3	4.5 ± 1.2	2.7 ± 0.7	70 ± 15	33 ± 8
D	120 ± 30	ND	0.43 ± 0.10	17 ± 4	2.7 ± 0.7	5.0 ± 1.0	45 ± 10	10 ± 3
E	160 ± 40	9 ± 2	0.59 ± 0.14	12 ± 3	3.8 ± 0.9	8 ± 2	75 ± 20	19 ± 5
F	33 ± 8	7.5 ± 1.8	0.39 ± 0.09	10 ± 3	3.6 ± 0.9	6.0 ± 1.5	44 ± 11	16 ± 4
1D	45 ± 10	11 ± 3	0.41 ± 0.10	11 ± 3	4.4 ± 1.1	1.7 ± 0.4	60 ± 15	5.5 ± 1.5
3D	27 ± 7	5.5 ± 1.5	0.33 ± 0.08	6.5 ± 1.5	1.2 ± 0.3	3.9 ± 1.0	33 ± 8	8 ± 2
5D	65 ± 15	2.7 ± 0.7	0.34 ± 0.08	10 ± 3	3.8 ± 0.9	2.7 ± 0.6	55 ± 14	14 ± 4
7D	23 ± 6	8 ± 2	0.33 ± 0.08	5.1 ± 1.0	2.5 ± 0.6	1.9 ± 0.5	54 ± 15	19 ± 5
8D	39 ± 9	13 ± 3	0.44 ± 0.11	14 ± 4	8 ± 2	5.0 ± 1.5	56 ± 16	15 ± 4
CNT	66 ± 14	13 ± 3	0.44 ± 0.10	7.0 ± 1.5	2.1 ± 0.5	2.6 ± 0.6	65 ± 15	12 ± 3
NCT AP								
B	230 ± 60	6.5 ± 1.5	0.31 ± 0.08	5.0 ± 1.1	3.3 ± 0.8	3.7 ± 0.4	44 ± 10	17 ± 4
C	130 ± 30	7.5 ± 1.9	0.31 ± 0.07	10 ± 3	3.3 ± 0.8	4.0 ± 1.0	61 ± 15	3.0 ± 0.7
D	100 ± 30	9 ± 2	0.32 ± 0.08	6.0 ± 1.4	1.3 ± 0.3	1.7 ± 0.3	43 ± 9	19 ± 5
E	100 ± 30	8 ± 2	0.30 ± 0.07	6.5 ± 1.5	ND	5.0 ± 1.5	29 ± 7	10 ± 3
F	100 ± 20	10 ± 3	0.35 ± 0.09	9 ± 2	ND	ND	50 ± 10	7.0 ± 1.5
1D	190 ± 50	12 ± 3	0.55 ± 0.15	7.1 ± 1.5	1.1 ± 0.3	3.1 ± 0.8	55 ± 15	12 ± 3
3D	100 ± 30	8 ± 2	0.45 ± 0.11	6.1 ± 1.6	1.5 ± 0.4	10 ± 3	46 ± 9	4.5 ± 1.0
5D	80 ± 20	6.0 ± 1.5	0.28 ± 0.07	3.3 ± 0.8	1.2 ± 0.3	2.2 ± 0.5	44 ± 10	5.0 ± 1.5
7D	170 ± 40	10 ± 3	0.36 ± 0.09	3.6 ± 0.9	2.2 ± 0.6	1.5 ± 0.4	45 ± 10	11 ± 2
8D	120 ± 30	9 ± 2	0.40 ± 0.10	3.5 ± 0.9	1.5 ± 0.4	2.1 ± 0.5	41 ± 9	11 ± 3
CNT	79 ± 19	11 ± 3	0.40 ± 0.10	5.5 ± 1.5	ND	1.6 ± 0.4	60 ± 15	14 ± 4
Factors								
T	***	***	***	***	***	*	NS	***
P	*	NS	***	***	***	***	**	***
Y	***	***	*	***	***	***	*	***
T x P	***	**	*	NS	NS	***	NS	***
T x Y	***	***	*	***	***	***	**	***
P x Y	***	***	NS	NS	**	***	***	***
T x P x Y	***	*	***	**	***	***	*	***

Abbreviations: m ± σ, mean ± standard deviation of three repetitions; BP, before pasteurization; AP, after pasteurization; ND, not detected; T, fermentation temperature; P, pasteurization; Y, yeast type; NS, not significant.
Significant at *P ≤ 0.05, **P ≤ 0.01 or ***P ≤ 0.001, respectively.

and 2% (*w/v*) sea salts (Vibrant SeaTM, Seachem Laboratories, Madison, GA, USA) at 28 °C under anaerobic conditions for 48–72 h using an anaerobic jar (Merck KGaA, Darmstadt, Germany).

The concentration of pure cultures of bacterial and yeast starters was determined as optical density at 600 nm by a Shimadzu UV-1800 spectrophotometer (Kyoto, Japan). The bacterial and yeast cultures were collected by centrifugation at 4000×*g* for 10 min, the pellets resuspended in peptone water and added to olives at a final concentration corresponding to about 8 Log CFU/mL and 7 Log CFU/mL, respectively. For each inoculated fermentation, a spontaneous one was

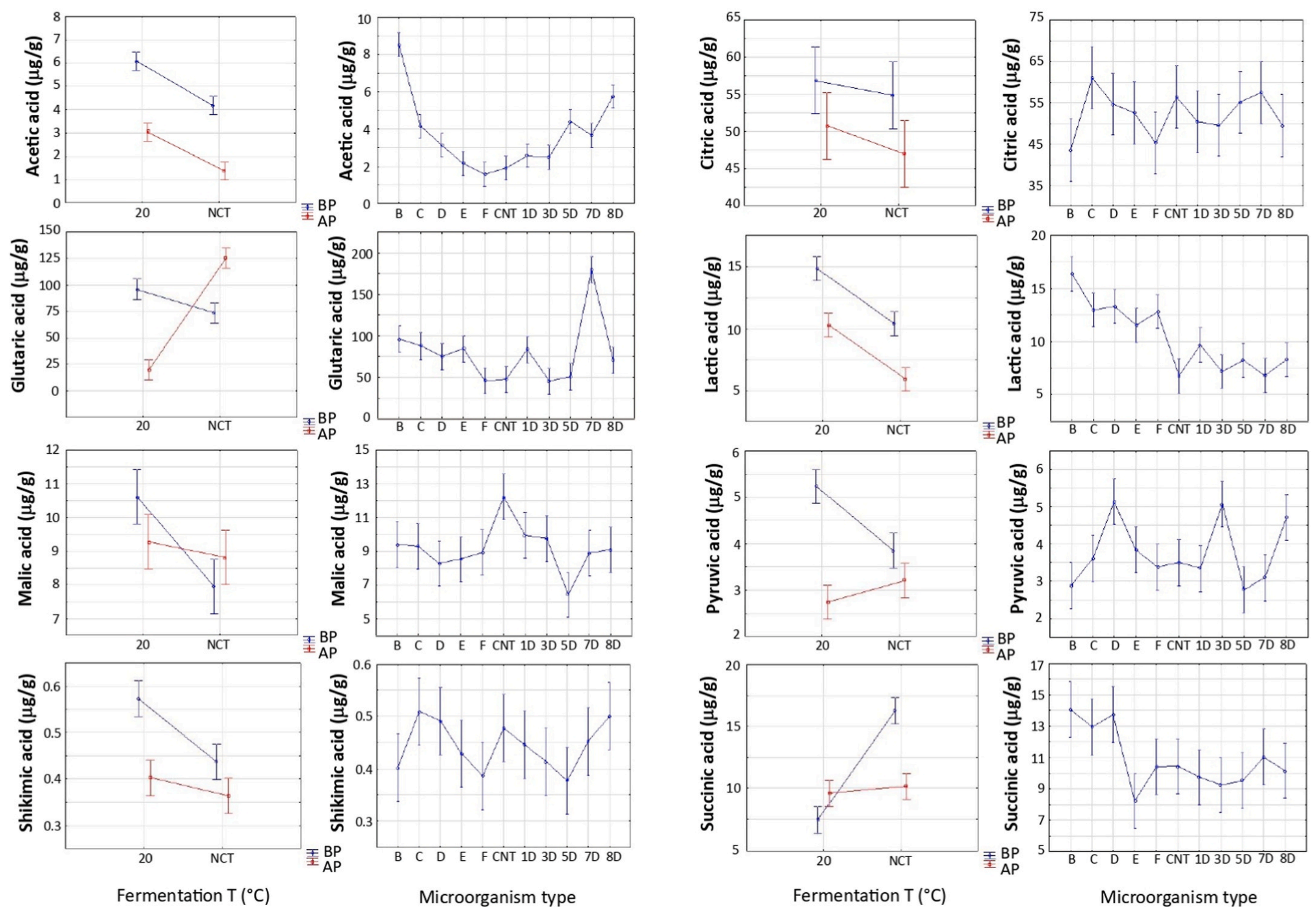

Fig. 2. Organic acids content produced by the fermentation of the samples with different microbial starters, treated at 20 °C and NCT, before (BP) and after pasteurization (AP) (data reported in Table 3).

carried out as control (CNT). Olive fermentation was considered ended when pH value was stable under 4.3 for at least 7 days and the olives were almost debittered, as assessed by at least three different persons tasting a sample in laboratory. In all tested conditions, the fermentations driven by microbial starters ended after 30 days incubation.

At the end of fermentation (30 days), before and after pasteurization, for the preliminary characterization of some sensory properties of the fermented table olives, a sensory panel of seven women and seven men (ranging from 30 to 70 years old) was used. The samples (5 olives for each test) were dispensed to the panellists in one session to select the best descriptors for several attributes, and a second session was performed to identify the intensity of the selected attributes/descriptors on a six-point intensity scale (0—none; 1-2—delicate; 3-4—moderate; 5-6—intense). The results were the mean values of the two sensory sessions.

2.1.3. Pasteurization conditions

At the end of the fermentation process, fermented table olives samples were washed with tap water, aliquoted in glass jars (each containing 0.5 Kg of olives and 0.25 L of brine NaCl 4% w/v and at pH 4 with citric acid). The pasteurization treatment was performed at 85 °C for 20 min using an industrial autoclave (Panini srl, Maranello, MO, Italy).

2.2. Microbiological analyses

Microorganisms present in brines of fermented olives samples were analyzed by serial dilutions with 0.1% (w/v) peptone water. After dilution, samples were tested onto agar plates containing: Man, Rogosa, and Sharpe Agar (MRS, LABM, UK) for LAB isolation in presence of 0.05 g/L of nystatin (Sigma-Aldrich, Darmstadt, Germany) and incubated at 30 °C for 48–72 h in anaerobic conditions; Plate count Agar (PCA, LABM, Heywood, Lancashire, UK) for the total bacterial count in presence of 0.05 g/L of nystatin (Sigma-Aldrich, Darmstadt, Germany) and incubated at 30 °C for 48–72 h; Violet Red Bile Glucose Agar (VRBGA, LABM, Heywood, Lancashire, UK) for Enterobacteriaceae enumeration (37 °C for 18–24h); Baird Parker Agar Base (Sigma-Aldrich, Darmstadt, Germany) supplemented with the rabbit plasma fibrinogen (Sigma-Aldrich, Darmstadt, Germany) for the enumeration of coagulase positive Staphylococci incubated at 37 °C for 24–48h; Violet Red Bile Agar (VRBA, LABM, Heywood, Lancashire, UK) for the detection and enumeration of coli-aerogenes bacteria incubated at 37 °C for 24–48 h; *Pseudomonas* agar (LABM, Heywood, Lancashire, UK) added with CFC supplement (LABM, Heywood, Lancashire, UK) incubated at 35 °C for 24–48 h, for *Pseudomonas* spp.; Sulphite-Polymyxin-Sulphadiazine Agar (SPS, Biolife Italiana srl, Milano, Italy) incubated at 35–37 °C for 18–48 h under anaerobic conditions, for the detection of *Clostridium* spp. Yeast total count was determined on Sabouraud dextrose agar medium (LABM, Heywood, Lancashire, UK) added with 0.1 g/L of ampicillin (Sigma-Aldrich, Darmstadt, Germany) and 0.05 g/L of kanamycin (Sigma-Aldrich, Darmstadt, Germany) at 30 °C for 3–4 days.

2.3. Compositional analyses

For the nutritional analysis, 10 g of drained or freeze-dried table

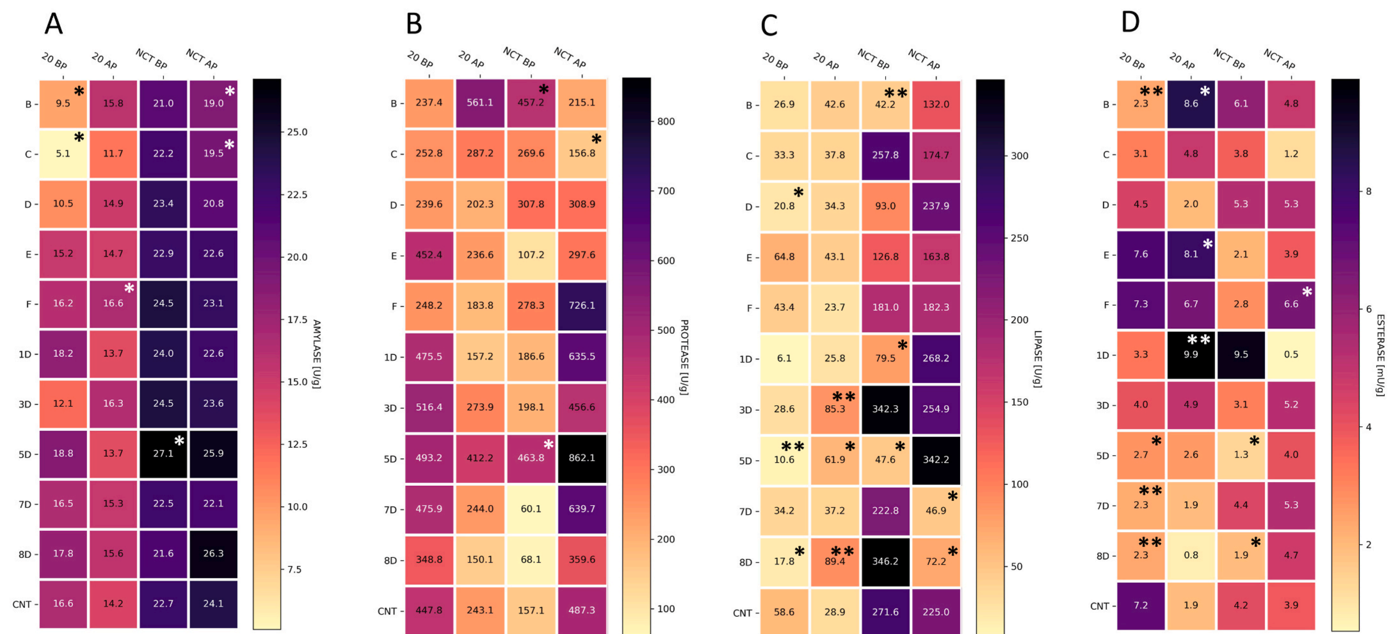

Fig. 3. Enzyme activities associated to fermented table olives samples. (A) amylase, (B) protease, (C) lipase and (D) esterase activities were reported for each sample fermented at 20 °C and at not controlled temperature (NCT), both before and after pasteurization. 20 BP: fermentation at 20 °C, before pasteurization; 20 AP: fermentation at 20 °C, after pasteurization; NCT BP: fermentation at not controlled temperature, before pasteurization; NCT AP: at not controlled temperature, after pasteurization. Data were submitted to one-way analysis of variance (ANOVA), Dunn's post hoc method was applied to compare each treatment with the control (**p* < 0.05; ***p* < 0.01).

olives were ground with a pestle and mortar in the presence of liquid nitrogen.

2.3.1. Sugar content

The extraction of the carbohydrates was carried out following the method described by Kahane et al. (2001) with some modification. Briefly, 100 mg of samples were suspended in 5 mL of a 20:80 water-ethanol (v:v) solution and incubated with shaking at 100 × *g* for 1 h at 30 °C. The mixture obtained was filtered through a Whatman filter paper grade 1 (Merck KgaA, Darmstadt, Germany), then the obtained extract was adjusted to 5 mL with 70% ethanol and concentrated in a rotary evaporator under reduced pressure and maintaining the temperature below 50 °C. Concentrated extracts were used for the Glucose and Sucrose Colorimetric Assay Kit (Sigma-Aldrich, Darmstadt, Germany) according to the manufacturer's instructions.

2.3.2. Protein content

The method described by Fleurence et al. (1995) was applied with slight modification. Concisely, 1 g of table olives powder was suspended in deionized water (5 mL) to allow cell lysis by osmotic shock and facilitate protein extraction. The suspension was gently stirred overnight at 4 °C. After incubation, the suspension was centrifuged for the first time at 10,000×*g* for 20 min and the supernatant was collected at 4 °C. The pellet was treated with 5 mL of 0.1 M NaOH in the presence of mercaptoethanol (0.5% v/v) (Sigma-Aldrich, Darmstadt, Germany). The mixture obtained was gently stirred at room temperature for 1 h before centrifugation at 10000×*g* for 20 min. The supernatant was then collected and combined with the supernatant of the first centrifugation. Subsequently, 0.1 mL of the obtained protein extract was added with 4 vol of cold acetone (−20 °C) mixed and incubated for 60 min at −20 °C. Subsequently, the suspension was centrifuged at 15,000 × *g* for 10 min. The supernatant was discarded, the obtained pellet was added with 400 μL of cold acetone (−20 °C) (Sigma-Aldrich, Darmstadt, Germany), and after mixing it was centrifuged at 15,000 × *g* for 10 min. The supernatant was discarded and the pellet was dissolved in 50 μL of buffer (2% NaCl, 0.4%NaOH, 0.16% Na/K tartrate, 1% SDS, all form Sigma-Aldrich, Darmstadt, Germany). Lowry Protein Assay according to the modifications by Harrington (1990) was used for protein quantification.

2.3.3. Ash content

Following the procedure described by Thiex et al. (2012), samples consisting of about 1.5 g fresh weight, were prepared into a porcelain crucible and placed in a temperature-controlled furnace. Ash content was determined by incineration at 550 ± 10 °C for 2h

$$\text{Residue on ignition (\% crude ash), \%} = (R - T) \times 100/(W - T).$$

where T = tare (empty) weight of crucible, R = weight of crucible + residue, and W = weight of crucible + test portion.

2.3.4. Lipid content

Total lipids were extracted using the modified method of Bligh and Dyer (1959) with some modifications (Ramires et al., 2022).

Dried samples (200 mg) were mixed with a total of 15 mL solvent added in this sequence: 6 mL of chloroform: methanol (2:1), 6 mL of chloroform: methanol (2:1) and 3 mL KCl (0.88%) (Sigma-Aldrich, Darmstadt, Germany). Samples were shaken for 15 s after the addition of each solvent and centrifuged at 5140×*g* for 5 min. The lower phase was set aside, and the upper phase was subjected to further extraction with a solution of chloroform: methanol (2:1, 1 V). The lower phase was isolated and added to the first one and mixed with a solution of methanol: water (1:1, 1/4 V). In this case, the lower phase was put aside, dried in the presence of nitrogen flux, and analyzed for total lipid quantification.

2.4. Organic acids extraction and HPLC analysis

Freeze-dried fermented table olives samples (in triplicate) (each of 500 mg) were macerated with 20 mL of methanol/water/formic acid (70:28:2) (Sigma-Aldrich, Darmstadt, Germany) at room temperature and incubated overnight at 4 °C in static mode. Samples were then centrifuged at 6500×*g* for 10 min. The supernatants were separated and the pellet was re-extracted with an additional 10 mL of solvent on a rotary shaker for 1 h at room temperature; finally, the supernatants were

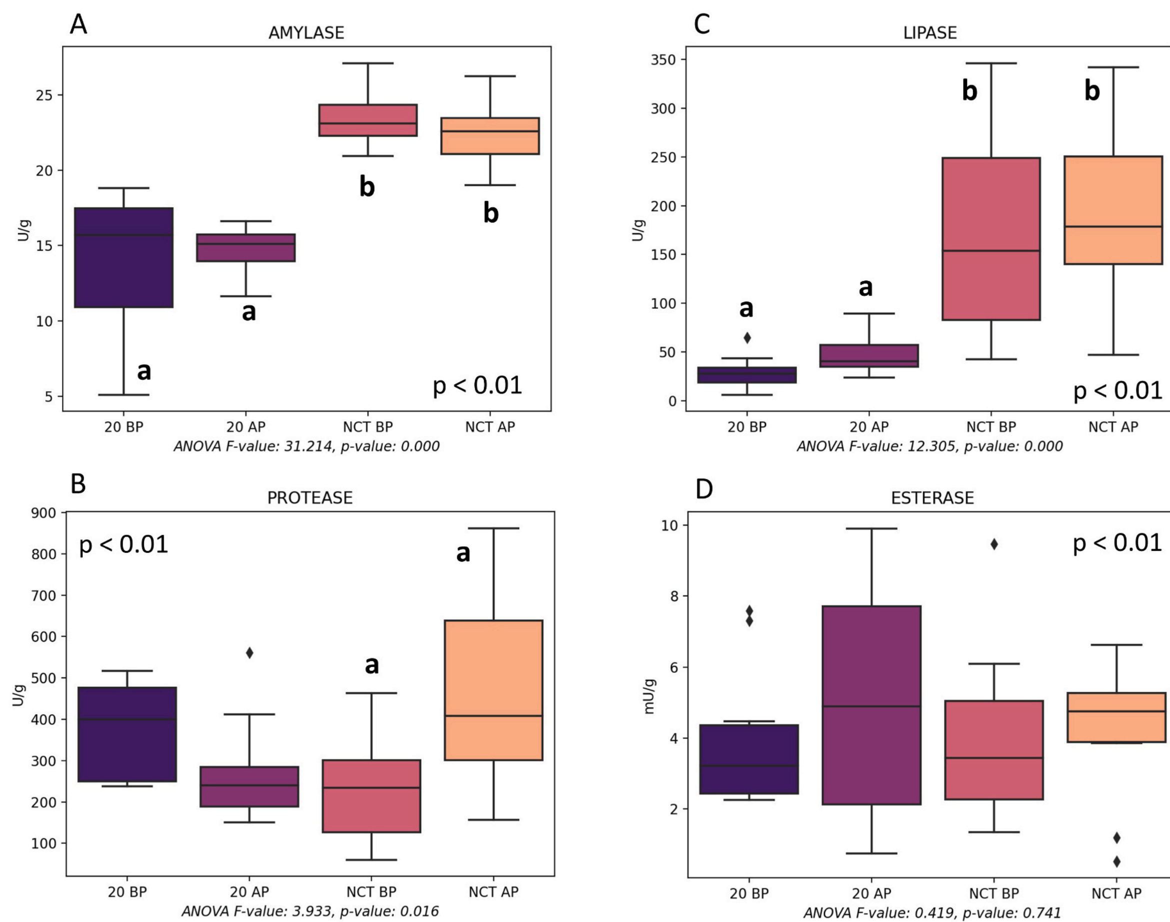

Fig. 4. Summary box-plot graphs of the enzyme activities associated to fermented table olives samples: (A) amylase, (B) protease, (C) lipase and (D) esterase activities content of fermented table olives in the two temperature conditions, before and after pasteurization. 20 AP: fermentation at 20 °C, after pasteurization; NCT BP: fermentation at not controlled temperature-before pasteurization; NCT AP: at not controlled temperature-after pasteurization. Data were submitted to one-way analysis of variance (ANOVA), Holm-Sidak post hoc method was applied to determine significant differences among samples (*$p < 0.01$).

combined, evaporated and reconstituted to 10 mL final volume in water 0.5% (v/v) formic acid for further analysis.

HPLC 1260 (Agilent Technologies, Palo Alto, CA, USA), composed of a degasser, quaternary pump solvent delivery, thermostat column compartment and diode array detector, was employed for the organic acid analysis. The extracts (3 μL) were injected onto a reversed stationary phase column, Zorbax Eclipse Plus-C18 (Agilent Technologies, Palo Alto, CA, USA) 5 μm (250 × 4.6 mm i.d.) operating at 20 °C with a flow rate of 0.8 mL/min. HPLC separation was carried out in isocratic mode.

with 0.1% aqueous formic acid. Stop time to 15 min. Diode array detection was between 190 and 400 nm, and absorbance was recorded at 210 nm.

Positions of absorption maxima (λ_{max}), absorption spectra profile, and retention times (RT) were compared with those from pure standards (analytical grade glutaric acid, malic acid, shikimic acid, lactic acid, acetic acid, pyruvic acid, citric acid, and succinic acid provided by Sigma Aldrich, Milan, Italy) and used for the organic acid identification. Organic acids quantification was performed by using the calibration curves in the concentration range of 1000–1 μg/mL, depending on the concentration of the different organic acids in the real samples, of glutaric acid ($R^2 = 0.9951$; LOD = 10.4 μg/mL; LOQ = 34.6 μg/mL), malic acid ($R^2 = 0.9956$; LOD = 9.8 μg/mL; LOQ = 30.7 μg/mL), shikimic acid ($R^2 = 0.9995$; LOD = 1.6 μg/mL; LOQ = 5.0 μg/mL), lactic acid ($R^2 = 0.9954$; LOD = 10.2 μg/mL; LOQ = 34.1 μg/mL), acetic acid ($R^2 = 0.9978$; LOD = 6.6 μg/mL; LOQ = 22.5 μg/mL), and citric acid ($R^2 = 0.9969$; LOD = 9.4 μg/mL; LOQ = 31.4 μg/mL). The detection limit (LOD) and quantification limit (LOQ) were calculated on the basis of chromatograms and defined as signal-to-noise (six times SD of baseline) ratio of 3 and 10, respectively.

2.5. Enzymatic activity assays

Activity assays for α-amylase, protease, esterase, and lipase were carried out in the liquid portion of the samples. All experiments were conducted in triplicate. For crude enzyme solution preparation, an aliquot of each fermented and unfermented sample was filtered by polyamide filter 355/51 (Saati, Milan, Italy), and the resulting liquid fraction was centrifuged at 15000×*g* for 15 min at 4 °C and the resulting supernatant was used for the assays.

2.5.1. α-Amylase activity assay

The α-amylase assay was performed as described by Ramires et al. (2022). Briefly, the reaction mixture consisted of 50 μL substrate solution (1% potato starch in pH 7 phosphate buffer), 93 μL of phosphate buffer at pH 7, and 72 μL of raw enzyme solution. One Unit (U) of activity of α-amylase was defined as the amount of enzyme required to release 1 μmol of glucose reducing-sugar equivalents per minute.

2.5.2. Lipase activity assay

A spectrometric method was performed for the measurement of lipase activity using *p*-nitrophenyl palmitate (*p*-NPP) (Sigma-Aldrich, Darmstadt, Germany) as a substrate (Park et al., 2007). The assay was performed on 1 mL of crude enzyme solution properly diluted as

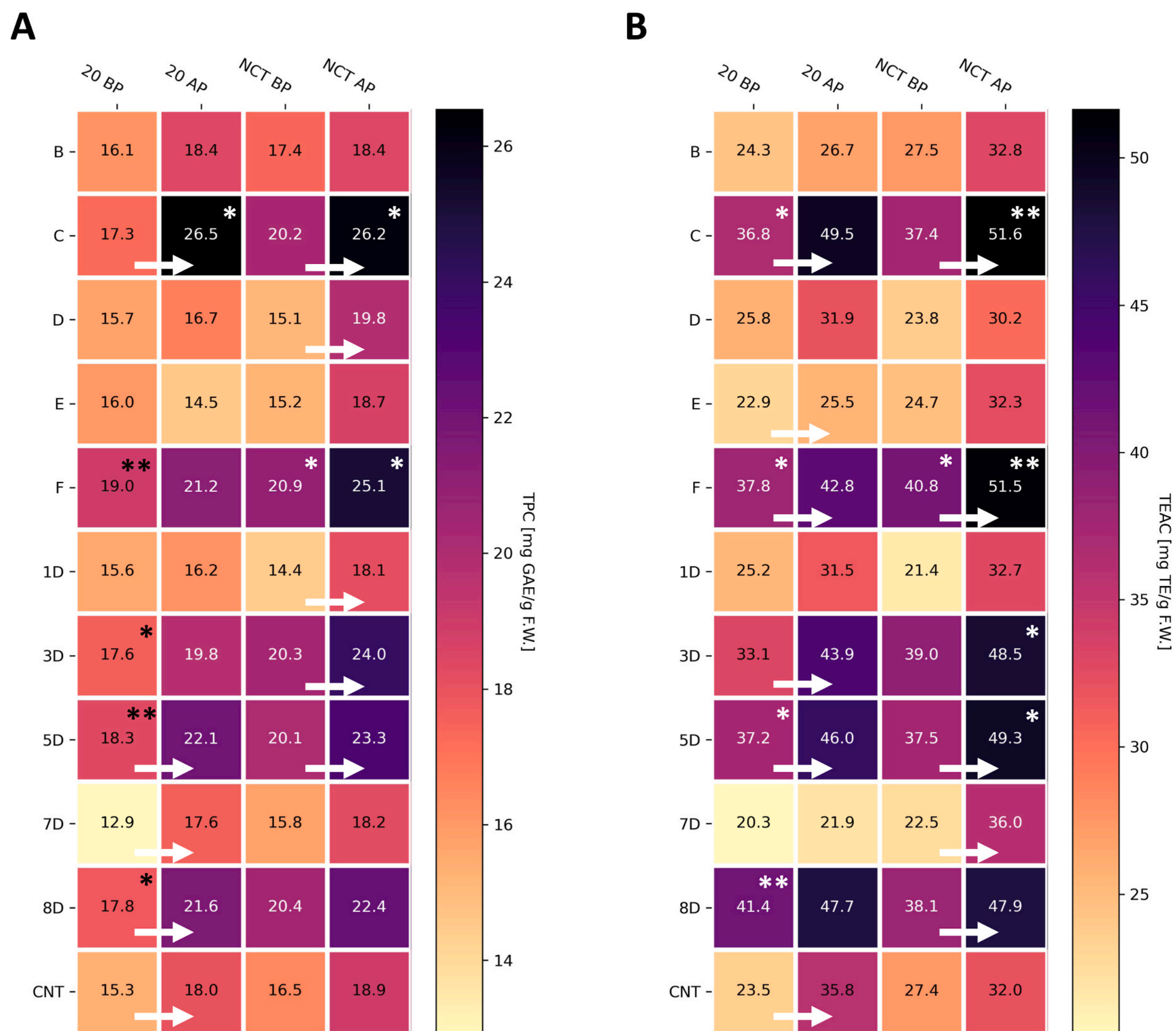

Fig. 5. Total polyphenols content and antioxidant activity associated to fermented table olives samples. A. Total polyphenol content (TPC) in table olive samples fermented at a constant and controlled temperature of 20 °C. B. Total antioxidant activity (AAT, expressed as Trolox Equivalent Antioxidant Capacity, TEAC) associated to table olive samples fermented at environmental not controlled temperature (NCT). 20 BP: fermentation at 20 °C-before pasteurization; 20 AP: fermentation at 20 °C-after pasteurization; NCT BP: fermentation at not controlled temperature-before pasteurization; NCT AP: at not controlled temperature-after pasteurization. Data were submitted to one-way analysis of variance (ANOVA), Dunn's post hoc method was applied to compare each treatment with the control (*$p < 0.05$; **$p < 0.01$). The corresponding pairwise matrices of comparison among the samples were reported in Fig. S1. The white arrows indicate samples where a statistically significant differences in terms of TPC and AAT (expressed as Trolox Equivalent Antioxidant Capacity, TEAC) were obtained before and after pasteurization treatment. Statistically significant differences between each sample before and after pasteurization was determined by the two sample-t-test (Fig. S2).

described by Maiorano et al. (2022). One unit (U) of lipase activity was defined as the amount of lipase required to release 1 μmol of *p*-nitrophenol from *p*-NPP in 1 min under the corresponding conditions.

2.5.3. Esterase activity assay

The carboxyl ester hydrolase (esterase) activity was determined using a modified spectrometric method (Lopes et al., 2011) following the hydrolysis of *p*-nitrophenylbutyrate (*p*-NPB, Sigma-Aldrich, Darmstadt, Germany) to *p*-nitrophenol at 37 °C for 5 min. One unit (U) of esterase activity was defined as the amount of esterase needed to release 1 μmol of *p*-nitrophenol per minute from *p*-NPB.

2.5.4. Protease activity assay

The method by Walter (1984) and Moyano et al. (1996), modified with the use of casein 0.66% (w/v) in 50 mM Tris-HCl buffer pH 8 as a substrate, as proposed by Sigma-Aldrich Company, was followed for this activity test. Protease enzyme activity was measured as a change in absorbance at 765 nm using the microplate reader Infinite M200 PRO (Tecan, Switzerland). One unit of enzyme activity was expressed as 1 μmol of tyrosine min^{-1} mg $protein^{-1}$.

2.6. Total polyphenol content and antioxidant activity determination

2.6.1. Folin-Ciocalteu assay

Total phenol content was determined following the method by Magalhães et al. (2010), using a microplate reader (Infinite M200 PRO, Tecan, Switzerland). Samples extracts (from section 2.4) (duly diluted, 50 μL), Folin-Ciocalteu reagent (Sigma-Aldrich, Darmstadt, Germany) diluted in Milli-Q water (1:5 v/v) (50 μL) were placed in each well of a microplate, then 100 μL of sodium hydroxide solution (0.35 M) were added. The absorbance value at 760 nm was recorded after 5 min of incubation. Gallic acid (Sigma-Aldrich, Darmstadt, Germany) was used to obtain a calibration curve in the range from 2.5 to 40.0 mg/L. The total phenol content was expressed as Gallic Acid Equivalents (GAE). The analyses were carried out in triplicate.

2.6.2. Antioxidant activity

Antioxidant capacities using the ABTS assay was evaluated in sample

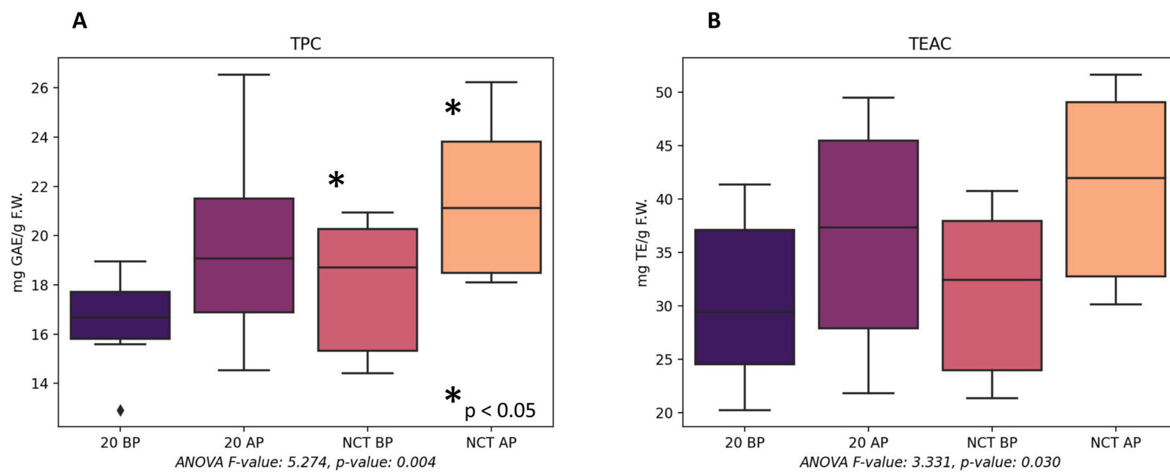

Fig. 6. Summary box-plot graphs of the total polyphenol content (A) and of the total antioxidant a (expressed as Trolox Equivalent Antioxidant Capacity, TEAC) (B) of fermented table olives in the two temperature conditions before and after pasteurization. 20 BP: fermentation at 20 °C-before pasteurization; 20 AP: fermentation at 20 °C-after pasteurization; NCT BP: fermentation at not controlled temperature-before pasteurization; NCT AP: at not controlled temperature-after pasteurization. Data were submitted to one-way analysis of variance (ANOVA), Holm-Sidak post hoc method was applied to determine significant differences among samples (*$p < 0.05$).

extracts (from section 2.4) as described in Blando et al. (2021). Briefly, Trolox (6-hydroxy-2,5,7,8-tetramethychroman-2-carboxylic acid; Hoffman-La Roche, Basel, Switzerland) was used as an antioxidant standard, and a 2.5 mM solution was prepared in Milli-Q water. ABTS (2, 2′-azinobis (3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt) and potassium persulfate (di-potassium peroxy-disulfate) were used to produce the ABTS radical cation. Sample extracts (duly diluted, 10 μL), Trolox standard at eight concentration (10 μL) and ABTS$^{\bullet}$ (200 μL) were incubated in each well and the absorbance at 734 nm was evaluated at the microplate reader (Infinite M200 PRO, Tecan).

All experiments were performed in triplicate.

2.7. Statistical analysis

All data represent the mean of at least three independent replicates (n = 3). Data are presented as mean values ± standard deviation. Statistical comparisons were performed developing scripts in Python using the following libraries: Pandas, Scipy, Numpy, Seaborn, Matplotlib, Sklearn.

For microbiological analyses, *t*-test assay was applied to compare differences between microbial counts; enzymatic activities, Kruskal-Wallis test followed by Dunn's multiple comparisons test were applied to establish significant differences among means values ($p < 0.05$). The mean values related to phenolic content and antioxidant activity were subjected to one-way ANOVA followed by Fisher's post-hoc test. Results analysis was considered as significantly for $p \leq 0.05$. Principal component analysis (PCA) was used to compare microbiological, chemical, and biochemical parameters associated with the fermented samples.

3. Results and discussion

3.1. Table olives fermentations

In this work, three different factors have been investigated for their influence on quality and nutritional traits of fermented black table olives (*Cellina di Nardò*): 1) selected microbial starters; 2) constant and controlled temperature (20 °C) versus ambient not controlled temperature (NCT); 3) pasteurization of the final product.

Regarding the first component, yeast and bacterial strains, previously isolated from several plant sources (such as fermented table olives, coffee, and miso, Table 1), were investigated. The main purpose was to assess their ability in improving the nutritional traits of black table olives submitted to a fermentation process, following the natural black olives method.

Among the investigated bacterial strains, four out of five were isolated from table olives fermentations and three belonged to the species *Lactiplantibacillus plantarum* (strains C 180-11, TB 11–32, and BC T3-35), whereas one strain belonged to the species *Leuconostoc mesenteroides* (strain KT 5-1). *L. plantarum* and *Lc. mesenteroides* strains are largely used in several fermented foods, especially vegetables, in presence of high salt concentrations (Tufariello et al., 2015; Chytiri et al., 2020; Tufariello et al., 2019; Koutsoumanis et al., 2020). Moreover, the bacterial strain *Staphylococcus pasteuri* SB42 was tested for the first time as an alternative and non-conventional starter for table olives production, since an important role for Staphylococci in the production of fermented food preparations has been reported recently (Devanthi and Gkatzionis, 2019; Ramires et al., 2022; Maiorano et al., 2022).

On the other hand, among yeasts, isolates from the species *Candida parapsilosis*, *Debaryomyces hansenii*, and *Saccharomyces cerevisiae* were tested. The presence of *C. parapsilosis* was reported in Italian, Algerian and Spanish table olive preparations (Aponte et al., 2010; Mourad and Nour-Eddine, 2006; Arroyo-López et al., 2016), as well as in other food fermentations (Aponte et al., 2010; De Melo Pereira et al., 2022; Mourad and Nour-Eddine, 2006; Ramires et al., 2022). Additionally, the non-conventional yeast strains *D. hansenii* A 15–44 and *Saccharomyces cerevisiae* LI 180-7 and KI 30-16, except for strain 10A, had already demonstrated their ability to achieve effective fermentation even in difficult conditions (Ramires et al., 2022; Tufariello et al., 2019).

As a second factor, the influence of two different temperature conditions on table olives fermentation was tested for 30 days: a controlled temperature maintained at the constant value of 20 °C; not controlled environmental temperature range of 7–17 °C, typical of the winter season (January in South East Apulia Region, Italy) (NCT). These two different conditions were chosen to test the most favorable strategy to promote interactions between the microbial starters and olive matrix, especially in terms of quality/safety traits and nutritional features of the fermented table olives. In fact, the effect of different temperatures (i.e., 25 °C, 18 °C, and room temperature), in presence of diverse NaCl levels in brines, were assessed for their influence on the microbiological and physicochemical characteristics of naturally black olives (Tassou et al., 2002).

As expected, different rates of bacteria and yeasts growth and survival were registered in the two tested incubation conditions (Table 2 A and B). Samples inoculated with bacterial starters and incubated at

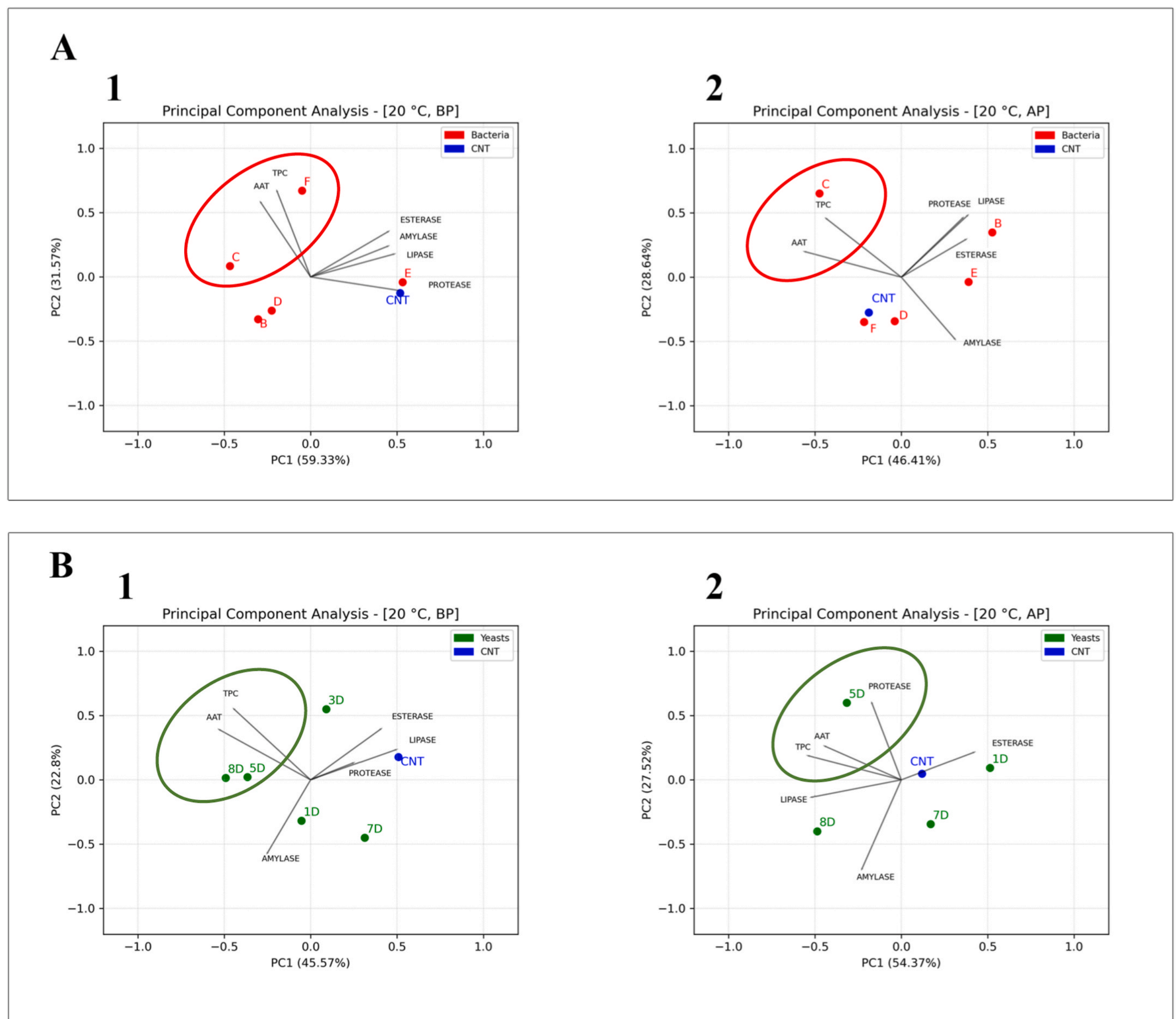

Fig. 7. Score scatter plot of PCA model performed on parameters associated with table olives samples fermented at 20 °C, before (Panel 1) and after pasteurization (Panel 2). PCA variables were the data obtained from the analysis of values of total phenolic content, antioxidant activity, enzyme-associated activities at the end of the process. A: samples inoculated with bacterial starters. B: samples inoculated with yeast starters. CNT: spontaneous fermentation, as control. BP: before pasteurization; AP: after pasteurization.

20 °C resulted in a total bacterial count and LAB total count several orders of magnitude higher than those detected in the corresponding samples fermented in NCT conditions. On the other side, the total levels of yeast and moulds were similar in both tested conditions. Regarding yeast-driven fermentations, samples incubated at 20 °C revealed a higher LAB count only (>1 order of magnitude), whereas TBC, yeast and moulds count values were almost similar in the two tested conditions. All the treatments also revealed absence of presumptive *Clostridium perfringens*, *Enterobacteriaceae* tested on Violet Red Bile Glucose Agar medium (VRBGA) and coliforms assayed on Violet Red Bile Agar medium (VRBA), staphylococci and *Pseudomonas* (data not shown).

The fermentation ended after 30 days in both temperature conditions as verified by the sensory analysis where the bitterness gustatory sensation reached low values or was almost absent (Table S1). This evidence can be probably attributable to the advanced ripening stage (middle of December) of the table olives used in this study. Among all samples before fermentation at both temperature conditions, the differences linked to some kinaesthetic descriptors, such as hardness, crunchiness and fibrousness were almost negligible (Table S1).

The third factor tested in this study was the thermal pasteurization. Its main purpose was to inactivate pathogens (Commission Regulation, 2007), although not specific guidelines and regulations for pasteurized vegetables are defined by the Authority. Therefore, the guidance for ready-to-eat foods on the market was used as a reference (Health Protection Agency, 2009). Among all tested microbial classes, a total absence of microorganisms in all the table olives pasteurized samples was achieved after a pasteurization at 85 °C for 20 min (data not shown). This evidence confirms data reported in a recently published paper (Jafarpour, 2022), where heat treatment of fermented green olives in similar conditions (85 °C for 15 min) produced a complete elimination of the initial count of total yeasts and moulds, mesophilic and thermophilic bacteria.

The pasteurization process did not affect the texture descriptors (hardness, crunchiness and fibrousness) in all tested samples (Table S1).

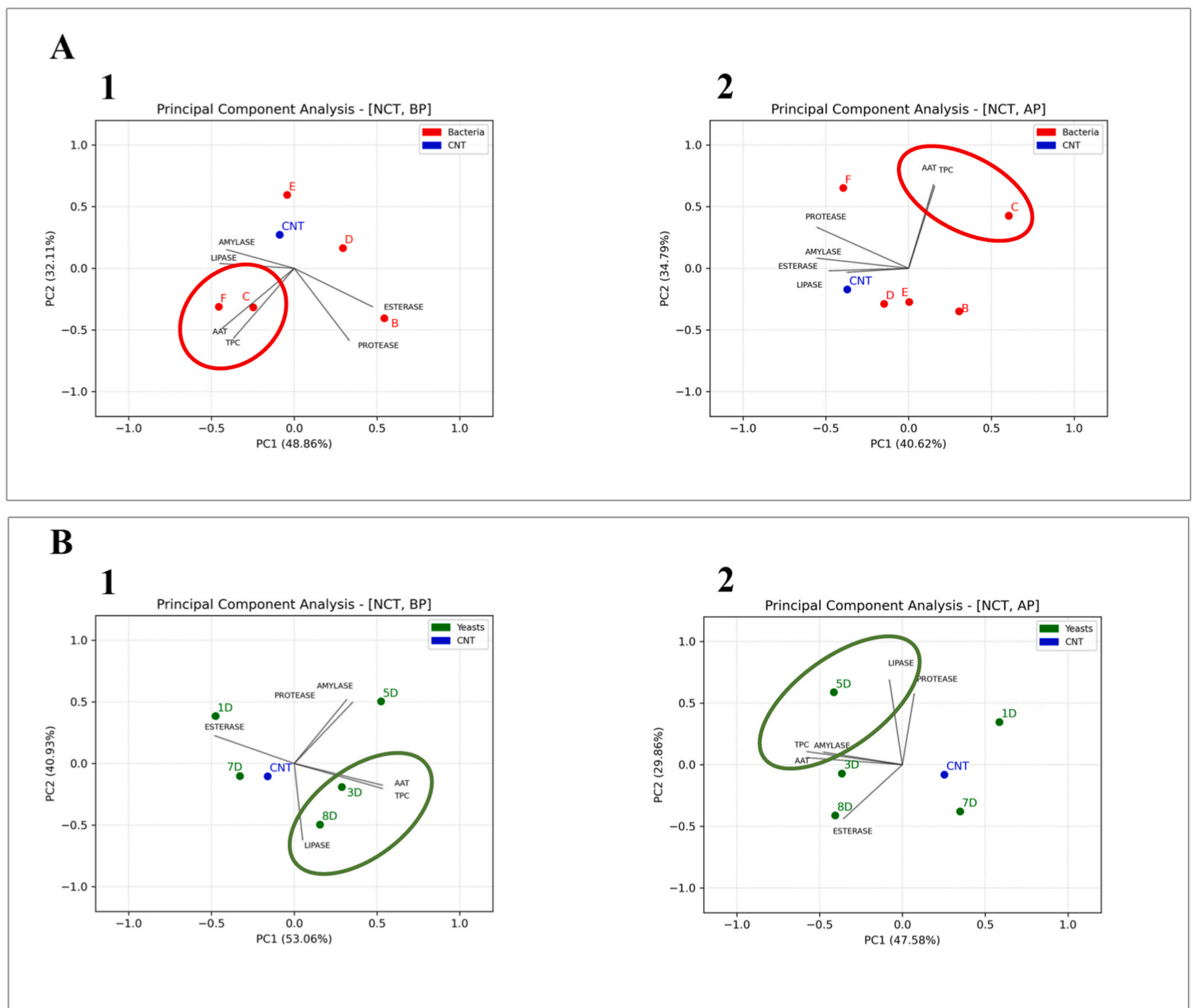

Fig. 8. Score scatter plot of PCA model performed on parameters associated with table olives samples fermented at not controlled temperature (NCT), before (Panel 1) and after pasteurization (Panel 2). PCA variables were the data obtained from the analysis of values of total phenolic content, antioxidant activity, enzyme-associated activities at the end of the process. A: samples inoculated with bacterial starters. B: samples inoculated with yeast starters. CNT: spontaneous fermentation, as control. BP: before pasteurization; AP: after pasteurization.

3.2. Composition analyses

Control samples of spontaneously fermented table olives were considered as reference standard material. Following their characterization, they showed that moisture, total fats, crude protein, ash, and total sugars values were within the range of values already reported in previous works (Sousa et al., 2011; Montaño et al., 2010; Unal and Negiz, 2003). In particular, moisture content was between 60.62 and 72.11 g/100 g FW; total fats between 21.31 and 31.9 g/100 g FW; total proteins between 0.44 and 0.57 g/100 g FW; total sugars between 1.92 and 2.44 g/100 g FW; ash content between 2.72 and 4.74 g/100 g FW (Table S2). Composition analysis was performed on the same samples before (BP) and after (AP) being submitted to pasteurization treatment. The main differences were observed in the protein and ash content (Table S2).

Within the samples treated at 20 °C, a common reduction of protein (−13% calculated as AP/BP %) and ash (−24%) content was observed among after the ones submitted to the pasteurization step. On the other hand, humidity, total fats, and sugar content was not affected, since variations were below 5% (Fig. 1, panel A). In the samples fermented at not controlled temperature (NCT), the pasteurization treatment produced a reduction in humidity (−11%), proteins (−10%), and ash (−6.5%), together with a significant increase in total fat content (>34%) (Fig. 1, Panel B). These results were different from what observed in *Azapa* black olives, where the pasteurization procedure (80 °C for 5 min) applied by Brazilian producers did not show significant changes in the content of the analyzed compounds, except for a reduction (4.1%) of moisture (Dias-Martins et al., 2020). Another study (Amooi et al., 2015) on four different table olive cultivars showed that pasteurization (90 °C for 15 min) did not produce significant differences in the fatty acid profiles. However, in the paper of Sánchez et al. (2017), the main differences were observed after the pasteurization treatment of fermented table olives, since this operation changed their volatile compounds composition.

Each sample of table olives showed a specific profile of organic acids composition, being influenced significantly by the combination of the

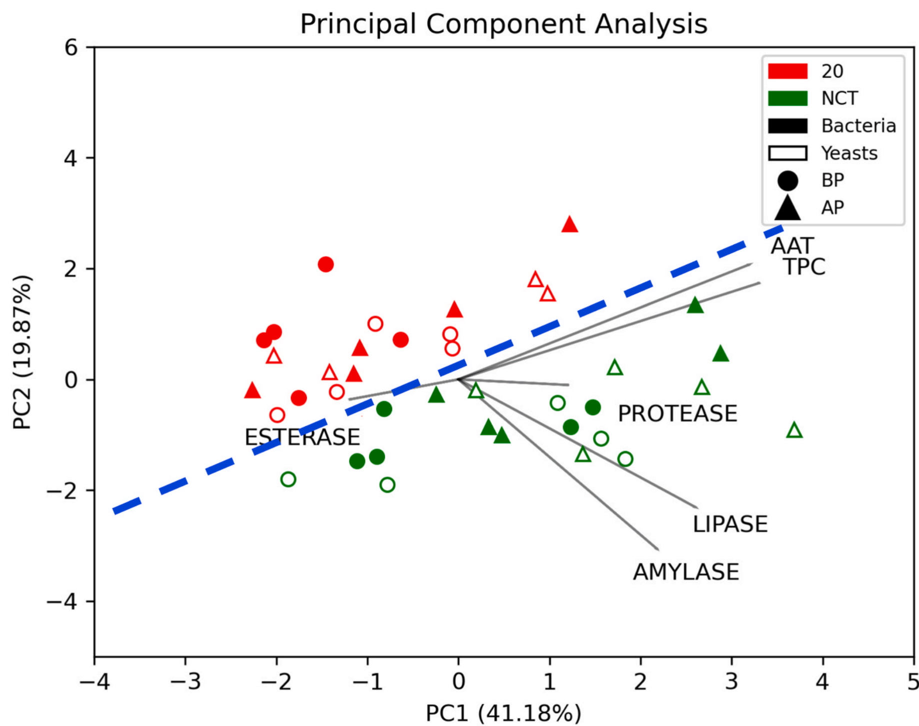

Fig. 9. PCA model performed on parameters associated with all table olives samples fermented with bacterial and yeast starters, at 20 °C (20) and not controlled temperature (NCT), before and after pasteurization. PCA variables were the data obtained from the analysis of values of total polyphenols content, antioxidant activity, enzyme-associated activities at the end of the process. BP: before pasteurization; AP: after pasteurization.

three tested factors (microorganism type, fermentation temperature and pasteurization) (Table 3). For the first time, this study also looked at how the organic acid composition is affected by applying either two different conditions of fermentation temperature or a pasteurization step on final products. As shown in Fig. 2, the microorganism type factor had also a significant impact on the organic acids composition. In any of the two conditions, at the end of the fermentation process, glutaric is the dominant organic acid, followed by citric (as it is influenced by external addition), succinic, lactic, and finally malic and acetic (with similar concentrations). As expected, the levels of lactic acid were higher in LAB fermented samples than in the yeast inoculated ones; samples B, C, D showed the highest concentration of succinic acid, and the highest level of acetic acid were also recorded in sample B. In general, pasteurization treatment and fermentation with not controlled temperature (NCT) caused a significant reduction of organic acids concentration, with the exception of glutaric acid, whose content was increasing in NCT samples submitted to pasteurization (Fig. 2). Moreover, it was observed that spontaneous fermentations (CNT) produced higher levels of malic acid only, compared to the starter-driven ones, in particular at 20 °C before pasteurization (16 ± 4 μg/mg).

The results reported in this paper are almost in agreement with the organic acids composition of olives and brines during and at the end of the fermentation process already described by several previous studies (Bleve et al., 2014; Tufariello et al., 2015; Hurtado et al., 2008). The high content of glutaric acid can be probably related to the conversion of the excess of citric acid resulting from the pathway of lysine biosynthesis in yeasts (Bhattacharjee and Tucci 1969). Citric acid is inherent in olive flesh: in this study though, its concentration was also affected by an external addition made during the preparation of the brines, both before fermentation and heat treatment, according to the company practices. Lactic and acetic acids result from microbial activity (Bleve et al., 2014) and their content is favorably appreciated since they not only contribute to the stability and preservation of table olives, but also play a key role thanks to their aroma and taste. The yeasts and heterofermentative LAB were also able to metabolize citric acid and generate acetic acid from fermentable material under environmental stress (Tzamourani et al., 2021). In addition, succinic acid levels are resulting from the microbial conversion of citric acid, also via a potential shift from hetero-to homo-fermentative metabolism of LAB (Reis et al., 2022).

3.3. *Enzyme activities*

As already reported by other studies, the registered enzyme activities correspond to the sum of the two components within each biological sample: the ones deriving from the vegetable tissues and the ones produced by the microbial counterpart (Maiorano et al., 2022; Ramírez et al., 2014). In addition, during the fermentation process, the enzyme stability and concentration can vary depending on temperature, pH, microbial consortia evolution, production of potential inhibitors, etc.

The enzymatic activities contribute to the flavor, texture, and safety traits of the final products, (Bonatsou et al., 2017; Perpetuini et al., 2020). In this study, four of the main enzyme activities were tested directly on extracts obtained by the fermented table olives samples.

Not controlled temperature condition (NCT) was able to improve amylase activities in all samples compared to the ones fermented at a constant temperature of 20 °C (Fig. 3, panel A, see also Table S3, and Fig. 4, Panel A). Moreover, the pasteurization process did not affect amylases, with the exception of samples B and C in NCT conditions, where a significant reduction was registered respect to the control sample.

Regarding protease activities after the pasteurization treatment, an opposite trend was registered in samples maintained at 20 °C and NCT: protease activities generally decreased in the former and increased in the latter ones (Fig. 4, Panel B). Moreover, the samples treated with yeast showed higher protease activities both before pasteurization when incubated at 20 °C (Fig. 3, Panel B, see also Table S3), and after pasteurization when fermented at NCT, even though a previous study reported that yeast isolates showed weak or null protease activity (Oliveira et al., 2017).

Low levels of lipases were reported in the samples fermented at 20 °C

(Fig. 3, Panel C, see also Table S3 and Fig. 4, Panel C). On the contrary, high lipase levels were registered in most part of the samples incubated at NCT, while after the pasteurization treatment each sample showed a specific behavior (Fig. 3, Panel C, see also Table S3).

In the olive samples fermented at 20 °C, esterase activities were mostly unaltered after pasteurization (Fig. 3, Panel D), whereas heat treatment produced a generalized increase in the samples fermented at NCT (Fig. 3, Panel D, see also Table S3 and Fig. 4, Panel D). Esterase and lipase activities are considered essential to final olive taste, since they can improve it by originating esters from free fatty acids and by changing the free fatty acids composition of the final product (Bautista-Gallego et al., 2011).

3.4. Total polyphenols content and antioxidant activity

Polyphenols present in edible parts of fruits and vegetables are generally in soluble-free or conjugated forms and the bound polyphenols account for an average of 24% of the total polyphenols (Acosta-Estrada et al., 2014).

Fermentation approach on table olives, inoculated with selected microbial starters, was applied to enhance the release of polyphenols as well as to improve their consequent antioxidant activity. Fig. 5 shows the total polyphenols content (TPC) and total antioxidant activity (AAT) of different table olives sample. Each of these samples was subjected to a fermentation at two different temperatures and to pasteurization. By column, the starter inoculated samples were compared with the control (spontaneous fermentation, CNT) and indicated by asterisks when statistically different. The matrices of these comparisons were fully described in the Supplementary Fig. S1.

As already reported in other studies, the fermentation process can increase the total polyphenols release from the olives, especially by using selected starters in comparison with commercial products (D'Antuono et al., 2018) and conventional fermentation (Caponio et al., 2019). In fact, during the fermentation of several agri-food materials, microbial hydrolytic enzymes (amylase, protease, β-glucosidase, xylanase, cellulase) can modify cell wall components. They can also facilitate phenolic unbinding and evolution by esterification, driven by esterase and lipase (Ramires et al., 2023; Dey et al., 2016; Hur et al., 2014) together with hydrolytic enzymes, laccases and phospho-lipases present in the plant cell wall (Puri et al., 2012).

The results reported here differed from those obtained in a previous paper. In particular, when studying *Cellina di Nardò* table olives fermented by indigenous bacteria and yeasts or selected microbial starters, D'Antuono et al. (2018) found a total polyphenols content 6 to 10 times lower than the one reported in this study (Fig. 5 and Table S4).

These differences can be explained in part by the advanced ripening state of the olives used at the time of the experiments (December 2020), and because the polyphenols amount was evaluated by Folin-Ciocalteu assay, method susceptible to interferences by others biological components present in the extract as reported in other studies (Bastola et al., 2017), respect to the HPLC-DAD analysis.

In this study, the use of microbial starters produced an improved recovery of polyphenols in almost all the samples of fermented table olives, independently on the temperature conditions in comparison to the spontaneous fermentation, used as control. In particular, regarding the microbial strains, the highest levels of total polyphenols were obtained in samples C and F, which were inoculated with bacterial strains; and in samples 3D, 5D, and 8D, which were driven by the presence of yeast starters instead, (Fig. 5, Panel A, 20 °C BP and NCT BP columns). These results were observed in both fermentation conditions and before the pasteurization step.

Fig. 5 also shows white arrows where significant differences in TPC were observed, before and after pasteurization (data reported by pairwise comparison in Supplementary Fig. S2). In particular, after pasteurization, a significantly improved level of these compounds was also achieved in some samples fermented at 20 °C (samples C, 5D, 7D, and 8D). Instead, the best results were observed after pasteurization in table olives samples C, D, and 1D, 3D and 5D fermented at NCT, with an increase of about 1.2–1.3 folds. In the remaining samples the pasteurization did not affect the initial (total polyphenols content) TPC.

As reported by other authors (Arfaoui, 2021), the consequences of thermal processing on polyphenols largely depends on the method applied. Heat damages cell walls, thus enabling the bound polyphenols to mobilize to other parts of the plant and enhancing their availability. At the same time, polyphenols are more prone to oxidation, and some of them are thermostable (Arfaoui, 2021). Furthermore, they can directly interact with intermediates during thermal processing (i.e., carbonyl-containing compounds) through Maillard reactions, lipid oxidation, and sugar condensation pathways that can play a positive or a negative role in food processing (Khan et al., 2020; Jafarpour, 2022). The decrease in total phenol content after thermal treatment was reported for *Carolea* brined olives (Piscopo et al., 2015) and for *Intosso* green olives (Marsilio et al., 2001).

In the two tested conditions of 20 °C and NCT, the total antioxidant activity (AAT) of olives samples at the end of fermentation process, was between 20.3 and 41.4 mg TE/g FW and between 21.4 and 39 mg TE/g FW, respectively (Fig. 5, Panel B, 20 °C BP and NCT BP columns). However, in all the inoculated samples produced by the two fermentation conditions described in this study, the increase of AAT can be derived by the positive impact of microbial activity on the table olives tissues. This results in an increase in the release of polyphenols, when compared to the spontaneous fermentation used as control (Fig. 5, Panel B, 20 °C BP and NCT BP columns, and Table S4).

After pasteurization, the levels of AAT surprisingly improved in all the table olive samples, independently from the fermentation temperature strategy. An increase between 1.2-1-4 times was observed in the samples C, F, 3D, 5D and 8D, which were inoculated with microbial starters and fermented at both temperatures (Fig. 5, Panel B, 20 °C AP and NCT AP columns, and Table S4).

At the end of fermentation and before pasteurization, the recorded levels of total antioxidant activity were in line with those reported in previous studies on different olive types: *Cellina di Nardò* table olives (D'Antuono et al., 2018); another *Leccino* black table olives (Caponio et al., 2019), fermented with spontaneous or starter-driven approaches; *Carolea* fermented olives and pasteurized at 70 °C for 18 min (Piscopo et al., 2015).

Finally, in accordance with other studies (Piscopo et al., 2015; D'Antuono et al., 2018), a correlation between the total phenols and the antioxidant activity can be found in treated samples, even though not exclusive contribution of phenols to the olive antioxidant value was hypothesized.

Indeed, no significant differences among total phenols content were observed in samples fermented at 20 °C and NCT (Fig. 6 Panel A), but a statistically significant increase in this value was obtained after the pasteurization of NCT fermented olives. Regarding the antioxidant activity of samples treated both at 20 °C and NCT, overall data did not show significant differences, even after the thermal processing (Fig. 6, Panel B).

3.5. Principal component analyses

Principal component analysis was successively applied to the main important biochemical parameters and nutritional traits associated with the fermented table olives samples. Bacterial and yeast treated samples were considered separately for both the fermentation temperature and pasteurization treatment, with the purpose of facilitating results interpretation and of identifying the optimal microbial strains as best performing starters.

The bi-plot is reporting table olives fermented at 20 °C (Fig. 7) with different bacterial and yeasts strains. The samples treated with the bacterial strains C and F were clustering in the plane with the two nutritional traits remarkable for our scope (TPC and AAT) (Fig. 7 A-1).

This behavior was confirmed only for the sample incubated with the bacterial strain C after the pasteurization step (Fig. 7 A-2). A similar view was also obtained for table olives fermented at the same temperature (20 °C) with the different yeast strains used in this study (Fig. 7 B-1). In this case, the samples 5D and 8D were correlated in plan with the two nutritional traits (TPC and AAT) (Fig. 7 B-1). Sample 5D was also confirmed as the best performing after the pasteurization process (Fig. 7 B-2).

The same approach applied to table olives samples fermented at NCT (Fig. 8) identified the ones better correlated with TPC and AAT values: C and F among bacterial treated samples before pasteurization (Fig. 8 A-1) and C sample after pasteurization (Fig. 8 A-2); 3D and 8D yeast treated samples before pasteurization (Fig. 8 B-1) and 5D after pasteurization (Fig. 8 B-2). In all the considered cases, these samples clustered in a portion of the plane clearly distinct from the corresponding position occupied by the spontaneous fermentation ones (CNT).

Finally, in order to obtain information about the possible role of the fermentation temperature and of the pasteurization on the table olive final products, a bi-plot considering all studied samples was produced (Fig. 9).

This approach revealed that all samples fermented at 20 °C were clustered together and located in a portion of the plane distinct from that one where NCT fermented samples were grouped. Moreover, all NCT samples, before and after the pasteurization process, were associated with the TPC and AAT nutritional traits.

4. Conclusions

In conclusion, the use of selected yeasts and bacteria for the fermentation of table olives led to a final product with nutritionally improved properties, increased amount of main polyphenolic compounds and, consequently, its total antioxidant activity.

In particular, among all microbial starters tested in this study, the bacterial strain C (*Leuconostoc mesenteroides* KT 5-1) and the yeast strain 5D (*Saccharomyces cerevisiae* LI 180-7) produced an interesting increase in total polyphenols content in both tested fermentation conditions and kept these properties almost unaltered even after the pasteurization process.

Data here reported suggest that fermentation of black table olives at a not controlled environmental temperature (NCT) could be more beneficial than using controlled and constant temperature conditions (20 °C), even though defining an optimal constant temperature was not in the scope of this work. At the same time, the absence of temperature control during the fermentation of table olives could be considered an important economic improvement for the process, as it is highly cost effective. Finally, the largely diffused pasteurization treatment of table olives seems playing a key role in enhancing the levels of the antioxidant compounds.

CRediT authorship contribution statement

Annamaria Tarantini: Writing – original draft, Validation, Methodology, Investigation, Conceptualization. **Pasquale Crupi:** Writing – original draft, Validation, Methodology, Investigation. **Francesca Anna Ramires:** Validation, Methodology, Investigation. **Leone D'Amico:** Methodology, Investigation. **Giuseppe Romano:** Methodology, Investigation. **Federica Blando:** Writing – review & editing, Supervision, Methodology. **Pierpaolo Branco:** Validation, Supervision. **Maria Lisa Clodoveo:** Writing – review & editing, Validation. **Filomena Corbo:** Writing – review & editing, Validation. **Angela Cardinali:** Writing – review & editing, Writing – original draft, Validation, Supervision, Funding acquisition, Conceptualization. **Gianluca Bleve:** Writing – review & editing, Writing – original draft, Validation, Supervision, Methodology, Investigation, Funding acquisition, Data curation, Conceptualization.

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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