Microbial dynamics and key sensory traits of laboratory-scale co-fermented green olives (Olea europaea L. cv. Ascolana tenera) and sea fennel (Crithmum maritimum L.)

Microbial dynamics and sensory traits of innovative high value preserves made by fermentation of started and not started table olives (*Olea europaea* L. cv. Ascolana tenera) with sea fennel (*Crithmum maritimum* L.)

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ABSTRACT

Table olives (*Olea europaea* L.) are one of the most important fermented vegetables worldwide, whereas sea fennel (*Crithmum maritimum* L.) represents an emerging food crop, characterized by interesting nutritional and sensory qualities. Both are characterized by a high concentration of bioactive compounds with health beneficial effects. Thanks to all these features, table olives and sea fennel undoubtedly represent two valuable ingredients for the manufacture of innovative vegetable-based preserves. Given these premises, the present study aimed to exploring the co-fermentation of table olives and sea fennel sprouts to produce laboratory-scale prototypes of innovative high value preserves. To this end, the use of a multiple strain starter was explored, together with Spanish style or Greek style processing method. The prototypes were hence evaluated for their microbial dynamics as well as key sensory traits by a panel of trained assessors. During the fermentation, all the prototypes showed a progressive pH reduction. Mesophilic lactobacilli, mesophilic lactococci, and yeasts represented the main microbial groups at the end of the fermentation, while Enterobacteriaceae decreased during fermentation. Metataxonomic analysis revealed an evolution of the microbiota, with *Lactiplantibacillus plantarum* dominating in all the prototypes in the late stage of fermentation, irrespective of the recipe, processing method and use of the starter. A greater crunchiness and lower fibrousness were perceived in the Greek style prototypes, which were preferred by trained panelists in respect with Spanish style prototypes.

KEY WORDS

Vegetable preserve; functional food ingredient; lactic acid bacteria; *Lactiplantibacillus plantarum*; metataxonomy; panel test

1. Introduction

Table olives are among the most important fermented vegetables all over the world, with an average 2016/17 - 2020/21 production attesting at 2.900 thousand tons (Committee for the Common Organisation of the Agricultural Markets - Arable crops and olive oil, 2022). The European Union is the main producer of table olives with an amount accounting at about one third of the total world production, followed by Egypt, Turkey, Algeria, and Morocco. Inside the European Union, the main producing country is Spain, followed by Greece and Italy (IOOC, 2022a; IOOC, 2022b).
According to the International Olive Oil Council (IOOC, 2004), table olives are prepared from the sound fruits of varieties of the cultivated olive tree (*Olea europaea* L.), treated to remove their bitterness and preserved by natural fermentation or, alternatively, heat treatment, with or without the addition of preservatives, and finally packed with or without covering liquid.
Table olives can be classified into three types, depending on the degree of ripeness of the fresh fruits, being: green olives, turning-color olives, and black olives (Rejano et al., 2010). The first type refers to fruits harvested during the ripening cycle prior to coloring, once that they have reached their normal size.
Two main methods are used for industrial-scale production of table olives, the Spanish (or Sevillean) style method and the Greek style method, the latter being also known as the "natural" method. The first method is articulated in the following phases: (i) deamarization of the olives by immersion in an aqueous sodium hydroxide solution (1.5 - 3.0 % NaOH, w v^{-1}) for 8 - 12 hours; (ii) washing to reduce residual alkali; (iii) fermentation and preservation in brine, an aqueous saline solution made with 8 - 10% NaCl wv^{-1}. In the Greek method, deamarization is carried out by placing the olives directly in brine; the duration of the process, which strictly depends on the degree of ripeness of the olives, requires at least 10 months of fermentation and storage (Corsetti et al., 2012; Rejano et al., 2010).
Lactic acid bacteria (LAB) are undoubtedly the main actors during table olives fermentation of, though these microorganisms compete with yeasts for the fermentation, with the latter microbial group potentially contributing to the flavor and aroma of table olives (Hurtado et al., 2012). The main activity of LAB during table olives fermentation is the release of organic acids, especially lactic acid, with a consequent pH drop and free acidity increase. Lactic acid produced by LAB inhibits the multiplication of spoilage and even pathogenic bacteria. Among LAB, leuconostocs, and streptococci are the least acid-producing, whereas homofermentative *lactobacilli* produce the highest amounts of lactic acid, followed by heterofermentative lactobacilli and pediococci (Hurtado et al., 2012).
Though a fast growth of LAB is required for a correct fermentation (Garrido-Fernández et al., 1997), the use of starter cultures is still debated; indeed, alongside authors who recommend the exploitation of inocula to control the fermentation and hence avoid defective products (Corsetti et al., 2012), numerous other authors have reported the successful fermentation of not started olives (De la Borbolla y Alcalá et al., 1964; Pelagatti and Brighigna, 1981; Balatsouras et al., 1983; Fernández-Díez et al., 1985; Montaño et al., 1993; Sánchez et al., 1995; Garrido-Fernández et al., 1997).

Fermented table olives represent a valuable Mediterranean healthy food, as well, thanks to their high content of bioactive compounds, dietary fibers, fatty acids, and antioxidants (Perpetuini et al., 2020; Campus et al., 2018; Boskou et al., 2015). In addition to this, they have been recognized as a source or even a vehicle of probiotic microorganisms (De Bellis et al., 2010; Bautista-Gallego et al., 2013; Bevilacqua et al., 2010; Blana et al., 2014; Peres et al., 2014).
Hence, they represent an ideal ingredient for the development of innovative vegetable-based foods, characterized by health-beneficial traits, balanced nutritional content, and pleasant sensory traits (Galanakis et al., 2021).
Given these premises, the present study was aimed at exploring the co-fermentation of Spanish and Greek style table olives (Olea europaea L.) and sea fennel (*Crithmum maritimum* L.) sprouts to produce started and not started laboratory-scale prototypes of innovative high value preserves. The latter were hence evaluated for their microbial dynamics as well as key sensory traits by a panel of trained assessors.
Green olives of the *Ascolana tenera* variety were selected taking into consideration the large size of the drupe, the very high flesh-to-stone ratio, and the low flash firmness, together with the acknowledged content of bioactive compounds, like polyphenols, phytosterols, monosaturated fatty acids, and tocopherols, able to reduce the risk of cancer and coronary heart diseases (Pannelli et al., 2001; Kailis and Kiritsakis, 2017; Kiai and Hafidi, 2014). Similarly, sea fennel was selected for its aromatic properties, thanks to the presence of essential oils, and crunchiness, but above all for the high content in bioactive compounds like essential ω-3 and ω-6 fatty acids, polyphenols, carotenoids, and vitamin C, able to prevent the development of chronic degenerative diseases (Maoloni et al., 2021; Generalić Mekinić et al., 2016).

2. Materials and methods

2.1 Microbial starter formulation

A multiple strain starter of lactic acid bacteria was used; it had previously been formulated with a pool of selected strains screened for key technological traits and exploited for fermentation of sea fennel sprouts in a brine salt solution (Maoloni et al., 2021). Briefly, it included 4 strains ascribed to the following species: *Lactiplantibacillus plantarum* (strain PB257)), *Leuconostoc pseudomesenteroides* (strain PB288), *Pediococcus pentosaceus* (strain FF78), and *Weissella confusa* (strain

PB321). All the strains belonged to the Culture Collection of the Department of Agricultural, Food, and Environmental Sciences (D3A, Università Politecnica delle Marche). They were stored at -80 °C in de Man Rogosa and Sharpe (MRS) broth (VWR, International, Radnor, Pennsylvania, USA) added with glycerol at a 3:2 ratio and subcultured in MRS broth (VWR) at 30 °C for 24 h, prior to their use.

2.2 Sea fennel and green olives supply and pre-treatment

Organic sea fennel sprouts (~ 6.5 Kg) were kindly supplied by a local farm (Azienda Agricola Paccasassi del Conero di Galeazzi Luca, Velieri Francesco e Babbini Alessandro, Ancona, Italy), which routinely cultivates sea fennel crop destined for the food industry. They were manually harvested in October 2019, washed in an aqueous hypochlorite solution (60 mg L^{-1}), rinsed in tap water, blanched at 95 °C for 30 s, and drained for 5 min using an industrial stainless steel vegetable strainer basket.

Green olives of the Ascolana tenera variety (*Olea europaea* L. cv Ascolana tenera) were kindly supplied by an industrial producer of table olives (Olive Gregori Società Agricola Semplice, Montalto delle Marche, Ascoli Piceno, Italy). Green olives were divided in two equal portions: one portion (~6 Kg) was subjected to an alkali treatment by submerging the olives in a 1.5 % NaOH solution (w v^{-1}), followed by rinsing with tap water, while the other portion (~6 Kg) was just washed with tap water without any alkali treatment. Both portions were drained 5 min with vegetable strainer baskets, prior to use.

2.3 Production of laboratory scale prototypes of fermented preserves

Laboratory-scale prototypes of fermented preserves were produced by mixing blanched sea fennel sprouts with treated or untreated green olives, according to: (i) recipe A, consisting of 10 % drained weight (dw) of sea fennel sprouts and 90 % (dw) of green olives; or (ii) recipe B, consisting of 60 % dw of sea fennel sprouts and 40 % dw of green olives. The fermentation was accomplished according to: (i) the Spanish style (S) method, using the green olives treated with alkali; or (ii) the Greek style (G) method, using the untreated olives, with or without (c, control) the inoculation of the multiple strain starter (s) to reach a final bacterial load of ~ 7 Log CFU mL^{-1} of brine. Four started laboratory-scale prototypes (SAs, SBs, GAs, and GBs) and four not started controls (SAc, SBc, GAc, and GBc) were then manufactured.

For each recipe (A or B) and each processing method (Spanish or Greek), three replicates were produced for both started and control preserves; each replicate consisted of 1.5 L glass jar filled with 750 mL of sterile brine (8 % w v^{-1} NaCl), previously autoclaved at 121 °C for 15 min, and 750 g of a mixture of sea fennel sprouts and green olives, at a ratio depending on the recipe (A or B). The fermentation was conducted at room temperature (18 ± 2 °C), for 63 (Spanish style method) or 373 (Greek style method) days, respectively.

The overall experimental plan is depicted in Figure 1.

2.4 pH measurement

Aliquots (1 mL) of brine were aseptically collected immediately after the inoculation (t_0) and during the fermentation process up until the end of the monitoring period, corresponding to 63 and 373 days, depending on the processing method, Spanish or Greek style, respectively (Fig, 1). The pH measurement was accomplished with a pH meter model 300 (Hanna Instruments, Padova, Italy). The results were expressed as the mean of three replicates ± standard deviation.

2.5 Microbial enumeration

For the microbiological analysis, additional aliquots (1 mL) of brine were aseptically collected immediately after the inoculation (t_0) and at selected intervals during the fermentation process (Fig. 1). Each aliquot was serially ten-fold diluted in sterile 0.1% (w v^{-1}) peptone water for the enumeration of: (i) mesophilic aerobic bacteria on Plate Count Agar (PCA, VWR), by incubating at 30 °C for 48 h; (ii) mesophilic lactic acid bacteria on De Man, Rogosa, and Sharpe (MRS) agar (VWR) supplemented with cycloheximide (VWR) (100 mg L^{-1}) to inhibit yeasts, by incubating at 37 °C for 48-72 h; (iii) mesophilic lactococci on M17 agar (VWR) supplemented with cycloheximide (VWR) (100 mg L^{-1}), by incubating at 22 °C for 72 h; (iv) yeasts on Rose Bengal chloramphenicol Agar (RBA, VWR), by incubating at 25 °C for 5 days; and (v) Enterobacteriaceae on Violet Red Bile Agar (VRBA, VWR), by incubating at 37 °C for 24 h. The results of viable counting were expressed as the mean Log CFU mL^{-1} of brine of three replicates ± standard deviation.

Aliquots of sea fennel sprouts and olives were aseptically collected from each replicate of the laboratory-scale prototypes at the end of their fermentation, using sterilized stainless steel tweezer; the collected samples were subjected to the enumeration of: (i) coagulase-positive staphylococci in accordance with the TEMPO:AFNOR BIO 12/28-04/10 standard method and (ii) sulfite-reducing bacteria according to the ISO 15213: 2003 standard method.

2.6 DNA extraction

Aliquots (1 mL) of the first decimal dilutions of selected brine samples (Fig. 1), prepared as described in section 2.5, were used for bulk cells preparations, by mixing the three dilution replicates of each sampling time. The mixtures were centrifuged at 14000 rpm to obtain cell pellets; these latter underwent total DNA extraction by using E.Z.N.A. soil DNA kit (Omega bio-tek, Norcross, GA, USA) according to the manufacturer's instructions.

2.7 Metagenomic analysis

Microbiota was analysed by amplifying the V3-V4 region of the bacterial 16S rRNA gene according to Klindworth et al. (2013) (16S Amplicon PCR Forward Primer 5' TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGCCTACGGGNGGCWGCAG; 16S Amplicon PCR Reverse Primer 5' GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGGACTACHVGGGTATCTAATCC. Library preparation and pooling were performed according to the 16S metagenomic sequencing library preparation from Illumina (Illumina, San Diego, CA). Paired end (2x250bp) sequencing was performed with a MiSeq instrument platform according to manufacturing instruction (Illumina, San Diego, CA). QIIME2 software (Callahan et al., 2016) was used for data analysis by following the quality filtering step of the dada2 denoise-paired plug in (Callahan, McMurdie, & Holmes, 2017) to obtain the Amplicon Sequence Variants (ASVs). Sequence variants with less than 10 reads in two samples were excluded from further analysis to increase the confidence of sequence reads and reduce bias by possible sequencing errors. Taxonomy assignment of ASVs was obtained through the qiime feature-classifier against the Greengenes database. Sequences of each ASVs were manually check by Basic Local Alignment Search Tool (BLAST) to confirm the taxonomic assignment. The ASVs tables, rarefied at lowest number of sequence/sample, displays the higher taxonomy resolution reached when the taxonomy assignment was not able to reach the species level, genus was displayed. The sequencing data were deposited in the NCBI Sequence Read Archive (SRA) and are available under the Bioprojects Accession Number PRJNA811759

2.8 Sensory analysis

At the end of the fermentation period, all the laboratory-scale prototypes were subjected to a sensory analysis carried out by a panel consisting of 10 non-smoker assessors, 5 males and 5 females, aged between 25 and 45, trained as described by Maoloni et al. (2021) with slight modifications. In more deatil, the panel was subjected to weekly training sessions, for three consecutive weeks, to identify the more suitable sensory attributes of both fermented greed olives of Ascolana tenera variety and sea fennel sprouts. The sensory analysis was performed in individual booths equipped with coffee beans for olfactory cleansing and still bottled water for oral rinsing before and between the evaluations (Resurreccion, 1998). The samples were coded with random, three-digit numbers and aliquots (10 g) of drained green olives and sea fennel sprouts were presented at room temperature to the panel. The panel was asked to separately evaluate sea fennel sprouts and green olives, based on the attributes previously set. In more detail, sea fennel sprouts were evaluated for: i) five olfactory descriptors, being herbal, woody, spicy, kerosene-like, and green olive; and (ii) five flavor descriptors, being herbal, woody, spicy, kerosene-like and green olive. Green olives were evaluated for: i) three olfactory descriptors, being green olive, spicy and kerosene-like and (ii) three flavor descriptors, being green olive, spicy, and kerosene-like. Occurrence of off-odors and off-flavors was also evaluated. Both green olives and sea fennel sprouts were evaluated for: i) four taste descriptors, being sour, bitter, salty, and sweet; and ii) three textural descriptors, being hardness, fibrousness, and crunchiness. For each ingredient (sea fennel or table olives) and each descriptor, panelists assigned a score comprised between 1 and 9, where 1 expressed the lowest and 9 the highest intensity. The panelists were also invited to express their global acceptance by using a 9-point hedonic scale, where 1 expressed the lowest (dislike extremely) and 9 the highest (like extremely) degree of liking (Peryam & Pilgrim, 1957). The results were expressed as means ± standard deviations.

2.9 Statistical analysis

The results of pH measurement, microbial enumeration, and sensory analysis were subjected to one-way analysis of variance (ANOVA) using JMP Version 11.0.0 software (SAS Institute Inc., Cary, NC, USA). The Tukey-Kramer honest significant difference (HSD) test ($P \leq 0.05$) was carried out to detect differences through multiple mean comparisons. Alpha diversity index was calculated by the diversity function of QIIME2 and analyzed by R-Studio software. Variables were compared by the non-parametric Wilcox test and visualized by R-studio as a box plot.

3 Results

3.1 pH measurement

The pH values measured in the prototypes during their fermentation are reported in supplementary Table 1, whereas the pH trends are shown in Figure 2. At t_0, the prototypes prepared according to recipe B showed significantly higher mean pH values (SBs: 6.14 ± 0.03; GBs: 6.82 ± 0.05) than the prototypes prepared according to recipe A (GAs: 5.75 ± 0.04; SAs: 5.89 ± 0.01), irrespective of the processing method applied. During the fermentation period, a progressive pH drop was seen in both starter and control (spontaneous) samples.

Regarding the Spanish style method, no significant differences were seen in the final mean pH values reached by the started (SBs: 3.47 ± 0.08) and control (SBc: 3.75 ± 0.27) prototypes made according to recipe B, whereas for the prototypes made according to recipe A, those started with the selected pool of lactic acid bacteria reached significantly lower mean values (SAs: 4.17 ± 0.05) than the control (SAc: 4.55 ± 0.02).

For the Greek style method, again, no significant differences were seen in the final mean pH values reached by the started (GBs: 3.42 ± 0.0) and control (GBc: 3.40 ± 0.04) prototypes made according to recipe B, whereas for the prototypes made

according to recipe A, those started with the selected pool of lactic acid bacteria strains reached significantly lower mean values (GAs: 3.88 ± 0.03) than the controls (GAc: 4.03 ± 0.03).

3.2 Microbial enumeration

The dynamic changes in the growth of specific bacterial groups are depicted in Figure 2, whereas viable cell counts are reported in supplementary Table 2.

Two similar trends emerged from the analysis of the prototypes processed according to the Spanish and Greek style methods, with some significant differences.

For mesophilic lactobacilli, at t_0 significantly higher values were recorded in the starter prototypes than the controls, irrespective of the recipe. However, as early as after 24 h of fermentation, a drastic decrease in the viable counts of this bacterial group was seen in all the started prototypes. At the end of the monitoring period, mesophilic lactobacilli reached mean viable counts ≥ 5.6 Log CFU mL^{-1} in the Greek style prototypes, with no significant differences between the two recipes, whereas in the Spanish style prototypes, they reached mean viable counts ≥ 6.6 Log CFU mL^{-1} in SAs, SAc, and SBc and equal to 6.0 Log CFU mL^{-1} in SBs.

Concerning mesophilic lactococci, at t_0 comparable low counts were recorded in the prototypes prepared according to the same method, with counts two orders of magnitude higher in the Spanish style prototypes than those processed according to the Greek style method; this microbial group showed an increase during fermentation, up to mean counts ≥ 6.1 and 5.7 Log CFU mL^{-1} in the Spanish and Greek style prototypes, respectively.

For yeasts, no differences were seen in the initial mean counts by comparing the two recipes or the two processing methods, with an increase up to mean counts ≥ 6.1 Log CFU mL^{-1} in the Spanish style prototypes and up to 5.5 - 5.7 or to 4.9 - 4.4 Log CFU mL^{-1}, in the Greek style prototypes made according to recipe A and B, respectively.

Viable counts of mesophilic aerobic bacteria revealed a similar behavior as the one depicted for mesophilic lactobacilli. In more detail, for the Spanish style method, at t_0 mean counts were comprised between 4.1 ± 0.2 (SBc) and 6.6 ± 0.4 (SAs) Log CFU mL^{-1}, whereas at the end of the fermentation they ranged from 6.2 ± 0.1 (SBs t_{63}) and 7.4 ± 0.0 (SAc t_{63}) Log CFU mL^{-1}. For the Greek style method, mesophilic aerobic bacteria ranged from 1.7 ± 0.3 (GAc t_0) and 6.8 ± 0.2 (GAs t_0) Log CFU mL^{-1} at t_0 to 5.7 ± 0.0 (GAs t_{373}) and 5.9 ± 0.0 (GAc t_{373}) Log CFU mL^{-1} at the end of the fermentation, respectively.

Enterobacteriaceae disappeared in all the prototypes at the end of their fermentation, except for SAc, where mean viable counts equal to 2.9 Log CFU mL^{-1} were detected. As a general trend, a faster reduction of this microbial group was observed in the started prototypes in respect with the control prototypes.

Coagulase-positive staphylococci and sulfite-reducing bacteria were under the detection limit (< 1 Log CFU g^{-1}) in all the prototypes.

3.3 Metataxonomic analysis

Figure 3 shows an overview of the ASVs detected by metataxonomic analysis, irrespective of the sampling time and the recipe. In the Spanish style prototypes of fermented preserves, *Lactiplantibacillus plantarum* (29% of the relative frequency), *Enterobacter cloacae* (30%), *Pediococcus pentosaceus* (7%), *Weissella confusa* (7%) and *Citrobacter freundii* (10%) were the main taxa. Except for *Citrobacter freundii*, the same microorganisms were found in the prototypes processed according to the Greek style method, with some differences; indeed, in the latter prototypes, *Lactiplantibacillus plantarum* was detected with a higher relative frequency (53%), whereas *Enterobacter cloacae* was among the minority taxa (6%). Moreover, in the Greek style prototypes, *Leuconostoc pseudomesenteroides*, added as a starter culture, was also detected, but at a relative frequency lower than 1%.

Figure 4 shows the bacterial dynamics assessed during the fermentation process of the Spanish style (**panel a**) and Greek style (**panel b**) prototypes, irrespective of their recipe, with *Lactiplantibacillus plantarum* being the dominant ASVs in the prototypes manufactured according to both the processing methods at the end of fermentation (**Figure 4 panel a and b**).

In the prototypes processed according to the Spanish style method, an evolution of the microbiota was seen during the fermentation process, with a stabilization in the late stage (**Fig. 4, panel a**). At t_0, the control (spontaneous) prototypes showed a higher heterogeneity of the microbiota than the inoculated prototypes. However, a very similar composition of the bacterial community was seen at the end of the fermentation process, in both the inoculated and control prototypes (**Figure 4 panel a**). Even evaluation of alpha diversity showed an increase in both the Chao1 index and the number of observations up until t_{10} followed by a stability over time, whereas the Shannon index highlighted an increase in the bacterial diversity from t_0 to t_{10}, followed by a sharp decrease in the last fermentation stage (**Supplementary Figure 1**). As a general trend, *Citrobacter freundii*, *Enterobacter cloacae*, *Lactiplantibacillus plantarum*, *Ralstonia* spp., and *Erwinia* spp., were the most abundant taxa (**Figure 4 panel a**). In more detail, *C. freundii* and *E. cloacae* increased up until t_5 and t_{10} respectively, whereas both decreased at the end of fermentation. *L. plantarum* increase at the end of fermentation (t_{35}, t_{63}), whereas *Ralstonia* spp. decreased during the fermentation process (**Figure 5 panel a**). As far as the recipe is concerned, no differences were seen between the Spanish style prototypes prepared according to recipe A or B

(**data not shown**). Moreover, in the started prototypes, the relative frequencies of *L. plantarum*, *L. pseudomesenteroides*, *P. pentosaceus*, *W. confusa* were higher than those of the control prototypes (**Supplementary Figure 2**).

In the prototypes processed according to the Greek style method, a similar trend to the Spanish style prototypes was seen, with an initial higher bacterial diversity of the control prototypes in respect with the started ones (**Fig. 4, panel b**). Again, a stabilization of the bacterial biota was seen in the late stage of fermentation ($t_{101} - t_{373}$), irrespective of the use of the starter. The alpha diversity of the Greek style prototypes showed a decrease of Chao 1 index, number of observations, and Shannon index during fermentation (**Supplementary Figure 3**).
L. plantarum, *Lacticaseibacillus zeae, Levilactobacillus brevis, P. pentosaceus*, and *Erwinia* spp. were the most abundant taxa (**Fig. 4, panel b**). In more detail, the relative frequencies of *L. plantarum*, *Lacticaseibacillus zeae* and *Levilactobacillus brevis* increased in the late stage of fermentation (from t_{101} to t_{373}), whereas *P. pentosaceus* decreased over time (**Figure 5 panel b**). As far as the recipe is concerned, ASVs related to *Erwinia* spp. and *L. plantarum* were more abundant in the prototypes containing the highest amount of sea fennel (recipe B) (**Supplementary Figure 4**). Moreover, significantly higher relative frequencies of *L. pseudomesenteoides*, *P. pentosaceus,* and *W. confusa* were seen in the started prototypes in respect with the control (spontaneous) prototypes ($P < 0.05$) (**Supplementary Figure 5**).

3.4 Sensory analysis

The results of the sensory analysis are depicted in Figure 6 panels a, b, c, and d. As a general trend, no faint off-odors or off-flavors were perceived in the assayed samples, irrespective of the recipe, the fermentation method, and the use of the starter. The tasting of sea fennel sprouts revealed hints of green olive in all the prototypes, except for SBs, together with herbaceous hints, again perceived in all the prototypes except for GAs and GBs. Going into green olives tasting, the kerosene-like scent was perceived in all the four prototypes (SBs, SBc, GBs and GBc) prepared according to recipe B, containing the highest percentage of sea fennel sprouts (60%). High scores for the salty descriptor and low scores for the sweet descriptor were assigned to both sea fennel sprouts and green olives. Regarding texture, sea fennel sprouts fermented according to the Greek style method were perceived as more crunchy and less fibrous than those fermented according to the Spanish style method. On the other hand, green olives fermented according to the Greek style method were perceived as more crunchy and harder than those manufactured according to the Spanish style method. Looking into the global acceptance, the prototypes prepared according to the Greek style method received a greater appreciation in respect with those manufactured according to the Spanish style method, with GAs and SBs gaining the highest (7.41 ± 0.08) and lowest (5.55 ± 0.09) scores, respectively.

4. Discussion

In this study, a multiple strain starter made of strains of lactic acid bacteria selected based on their pro-technological traits (Maoloni et al. in 2021) was exploited to guide the co-fermentation of mixtures of green olives and sea fennel sprouts in a brine salt solution. The starter was formulated including strains with different fermentative pathways, namely an obligate homofermentant (*P. pentosaceus* strain FF78), a facultative heterofermentant (*L. plantarum* strain PB257) and two obligate heterofermentants (*L. pseudomesenteroides* strain PB288 and *W. confusa* strain PB321). The composition of the starter was aimed at possibly support the establishment of a microbial succession during the fermentation process, with the homofermentative strains with a higher tolerance for acidic and anaerobic conditions dominating during the late fermentation stage. In fact, in cabbage-based products (e.g.: sauerkraut and kimchi), optimally fermented at 18°C with ~2% NaCI, a two-stage fermentation is established, with an initial dominance by heterofermentative lactic acid bacteria, including *Leuconostoc* spp. and *Weissella* spp., followed by a homolactic stage, where lactobacilli prevail (Snyder et al., 2021). By contrast, in fermentation of cucumbers, usually conducted at temperatures ≥ 20°C and NaCl content ≥ 6%, homolactic fermentation predominates, with *L. plantarum* being the prevailing species.

In the present study, the fermentation was conducted at 18°C with 8% NaCl. A temperature range between ~18 and 24°C has previously been reported to stimulate the metabolic activity of lactic acid bacteria, and hence production of organic acids and flavor enhancement (Snyder et al., 2021).

As far as pH evolution is concerned, at the beginning of fermentation, the prototypes prepared according to recipe B, containing 60% drained weight of sea fennel, showed significantly higher pH values than the prototypes containing 10% drained weight of sea fennel; by contrast, an opposite trend was seen at the end of fermentation, with significantly lower pH values measured in the prototypes manufactured according to recipe A in respect with recipe B.

In fermented vegetables, acidification mainly results from the accumulation of organic acids (especially lactic and acetic acid) produced by homo- or hetero-fermentative lactic acid bacteria from free sugars, primarily glucose and fructose (Snyder et al., 2020). In addition to this, during olives fermentation, alkaline hydrolysis of oleuropein results in the production of phenolic compounds, whose further breakdown leads to the release of additional acids, like elenolic acid (Kiai and Hafidi 2014).

In the present study, the more intense acidification observed in the prototypes with the highest sea fennel content might feasibly be related to the faster growth of lactic acid bacteria and hence a more intense production of organic acids in

presence of this aromatic herb. A similar behavior has already been described by Zaika and colleagues, in a study performed in 1983, where the effects of different aromatic herbs (oregano, rosemary, sage, and thyme) on growth and acidification of *Lactiplantibacillus plantarum* and *Pediococcus acidilactici* were evaluated. Intriguingly, after an initial inhibition, and hence a delay in fermentation, at sublethal concentrations, all the assayed herbs exerted a stimulation of the two lactic acid bacteria growth and acids production.

Going into the microbial enumeration, in the started prototypes, as early as after 24 hours of fermentation, mesophilic lactobacilli showed a drastic reduction, followed by a slight but continuous increase up until the late fermentation stage, whereas in the control prototypes a progressive growth of this microbial group was seen. Similar trends were observed by Maoloni et al. (2021), while fermenting sea fennel sprouts started with the starter culture formulation assayed in the present study. The early reduction of viable counts of mesophilic lactobacilli seen in the all the started prototypes irrespective of the processing method might be tentatively ascribed to the low competitiveness and hence adaptation ability of the inoculated strains in the specific substrate assayed. As it has been underlined above, for various aromatic herbs a stimulation of the lactic acid bacteria growth has been observed, after the bacteriostatic activity is overcome, (Zaika et al., 1983). To date, numerous studies have reported the antimicrobial effect of sea fennel essential oils (Senatore et al., 2000), but no data are available on a possible growth stimulation of lactic acid bacteria by this aromatic herb.

Regarding the control prototypes, dynamics of mesophilic lactobacilli were in accordance with the available literature on green olives fermentation (Marsilio et al., 2005; Panagou and Tassou 2006; Sánchez et al., 2000).

As far as the enumeration of Enterobacteriaceae is concerned, the growth dynamics emerged from the present study were comparable to those previously reported by other authors in either fermented table olives (Panagou and Tassou 2006; Hurtado et al., 2008) or sea fennel (Maoloni et al., 2021), with an initial multiplication of these microorganisms followed by a decrease up until a complete dye off at the end of the fermentation period, as a direct consequence of the pH drop due to lactic acid bacteria metabolism (Hurtado et al., 2008; Botta and Cocolin 2012).

For yeasts, despite initial (t_0) low loads, an overall significant increase of viable counts was seen during fermentation; indeed, at the end of the monitoring period, viable counts of yeasts were comparable to those of mesophilic lactobacilli in all the prototypes, except for GBs and GBc, where yeasts were about one order of magnitude lower than mesophilic lactobacilli. A similar trend has already been described by monitoring the microbial dynamics during sea fennel pilot scale fermentations (Maoloni at al. 2021). Regarding green olives, a higher load of yeasts in respect with mesophilic lactobacilli was seen at the end of fermentation of unstarted Greek style olives (Marsilio et al., 2005). Various detrimental properties have previously been associated with yeasts in olive fermentation, including: (i) antagonistic effect against lactic acid bacteria; (ii) fruit damage by excessive CO_2 production; (iii) degradation of polysaccharides of the olive cell wall through a polysaccharolytic activity; (iv) softening of the fruit during storage through a polygalactunorase activity (Arroyo-López et al., 2008; Arroyo-López et al., 2012). However yeasts can also carry out some beneficial activities, including: (i) production of key aromatic compounds such as glycerol, esters, ethanol, higher alcohols and other volatile compounds; (ii) synthesis of bioactive compounds with antioxidant properties; (iii) synthesis of killer toxins active against spoilage microorganisms and human pathogens; (iv) degradation of phenolic compounds; (v) improvement of lactic acid bacteria growth through the synthesis of vitamins, purines, and amino acids or the breakdown of complex carbohydrates (Arroyo-López et al., 2008; Arroyo-López et al., 2012).

The metataxonomic analysis clearly showed the occurrence of *L. plantarum*, *P. pentosaceus*, *W. confusa, E. cloacae*, and *C. freundii.* Members of the *L. plantarum* group, including *L. plantarum*, *L. pentosus*, and *L. paraplantarum*, are crucial microorganisms in table olives fermentation, with the first two species being also extensively exploited as starter cultures (Cocolin et al., 2013; Lucena-Padrós et al., 2015; Randazzo et al., 2012). In more detail, *L. plantarum* has been reported to dominate up until the end of table olive fermentation (Zago et al., 2013). The latter species is known to: (i) boost oleuropein breaking down due to its β-glucosidase activity; (ii) produce antimicrobial molecules against undesirable microorganisms; (iii) grow in a wide range of pH values and temperatures; and (iv) tolerate saline environments (Snyder et al., 2021). Starter cultures belonging to *L. plantarum* are known to improve the microbiological stability and safety of fermented table olives (Zago et al., 2013; Randazzo et al., 2012). In the present study, as expected, the starter species *L. plantarum* was found to quantitatively prevail in the inoculated prototypes, followed by the other inoculated species *L. pseudomesenteroides*, *P. pentosaceus*, and *W. confusa*. However, *L. plantarum* was also predominant in the uninoculated prototypes, thus demonstrating its occurrence on vegetable tissues and its high adaptation to the specific fermentation conditions applied for olive fermentation (Snyder et al., 2021).

Even *W. confusa* and *P. pentosaceus* are vegetable associated microorganisms, being often isolated from fermented vegetables (Hurtado et al., 2012; Quattrini et al., 2020). Though *W. confusa* is not included among the major genera used as starters, it is frequently isolated from spontaneous fermented foods, where it contributes to the characteristics of the products (Fessard and Remize, 2017). More specifically, *W. confusa* is known to produce high amounts of exopolysaccharides with texturizing properties, whereas some *Weissella* strains were found to decarboxylate polymeric

phenolic compounds, thus increasing bioavailability of these health beneficial compounds. Despite the above cited properties, the exploitation of *Weissella* spp. as a commercial starter is currently underexplored.

By contrast, *P. pentosaceus* is recognized as a promising lactic acid bacteria species for its application as adjutant or probiotic by the food industry, thanks to (i) the improvement of texture, sourness, and other organoleptic properties of foods; (ii) the antioxidant effects; (iii) the inhibition of pathogenic bacteria and fungi (Jiang et al., 2021).

Enterobacter and *Citrobacter* were both detected with relative high frequencies in the Spanish style prototypes, whereas the first genus was also found with a relative low frequency in the Greek style prototypes. Both these genera include opportunistic human pathogenic species, such as *E. cloacae* and *C. freundii,* whose occurrence in food products might represent a sanitary risk (Bevilacqua et al., 2010; Bevilacqua et al., 2013; Liu et al., 2018; Surowsky et al., 2014).

During the Spanish style fermentation, the plant-pathogenic genus *Erwinia* and the soil associated genus *Ralstonia* (Kado et al., 2006; Penland et al., 2020) were mainly detected in the early stage of fermentation, whereas in the late stage they were completely replaced by *L. plantarum*. This finding agrees well with what reported in the literature about the occurrence of a microbial succession during fermentation of vegetables, with epiphytic aerobic microbiota being replaced by facultatively anaerobic, acid-tolerant lactic acid bacteria (Snyder et al., 2021).

During the Greek style fermentation, the most significant detected species were *L. plantarum*, *Lacticaseibacillus zeae*, *Levilactobacillus brevis,* and *P. pentosaceus*. Among these, both *L. brevis* and *P. pentosaceus* have previously been detected in green table olives (Hurtado et al., 2012), whereas *L. zeae* is generally associated with dairy products (Poltronieri et al., 2008).

Going into the sensory analysis, as a general trend, a higher crunchiness was perceived by panelists by tasting sea fennel processed according to the Greek style in respect with the Spanish style method. Crunchiness undoubtedly represents a quality trait of sour vegetable-based fermented pickles, such as cucumbers and Capparis (Behera et al., 2020). Similarly, olives fermented according to the Greek style method were perceived as harder and crunchier than those processed according to the Spanish style method. These findings agree well with those previously reported by Marsilio et al. (2005) in a study dealing with the fermentation of green olives of the Ascolana tenera variety, processed according to both Spanish and Greek style methods. In fact, as it has previously been elucidated, the treatment of olives with lye in the Sevillian method leads to a softening of the olive flesh and hence to a modification of its texture (Chranioti at al., 2018). Furthermore, the greater overall acceptability of the Greek style prototypes in respect with the Spanish style prototypes might be tentatively ascribed to the prolonged fermentation process, with long-term fermentations being known to greatly contribute to the flavor and aroma of fermented products (Sabatini, & Marsilio, 2008).: In fact, the development of odors and flavors in a fermented food is related to the progressive accumulation of alcohols, acids, aldehydes, ketones, esters, sulfur compounds, terpenes and lactones from lactic acid bacteria and yeasts metabolism of carbohydrates, proteins, lipids, citrate, and polyphenols (Smid and Kleerebezem, 2014; Carballo et al., 2012).

5. Conclusions

Based on the results overall collected a few considerations can be made. The pH evolution was apparently affected by the recipe, with initial significantly higher and final significantly lower pH values of the prototypes with the highest sea fennel content. The inoculation of the starter culture had apparently no impact on the load of mesophilic lactobacilli, expect for the very early fermentation stage; similarly, in both the started and control prototypes, a complete dominance of the species *L. plantarum* was seen in the late fermentation stage, irrespective of the recipe, the use of the multiple strain starter, and the processing method. By contrast, this latter variable seemed to affect the final loads of mesophilic bacteria and yeasts, with significantly higher counts of both these microbial groups detected in the Spanish style rather than the Greek style prototypes.

As a general trend, a faster reduction of Enterobacteriaceae was observed in the started prototypes than the controls. In any case, a microbial succession was established during fermentation, irrespective of the recipe and processing method, with species dominating in the very first fermentation stage (e.g.: epiphytic bacteria) been replaced by acid resistant, facultative anaerobic microorganisms in the late stage. Finally, a higher crunchiness, a lower fibrousness, and a greater overall acceptability were scored in the Greek style prototypes than the Spanish style ones, with recipe A, characterized by the lowest sea fennel content, been preferred in respect with recipe B by the trained panelists.

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