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Deliverable title	D5.1 At least 2 pilot scale prototypes of sea fennel-based fermented shelf stable preserves
Deliverable Lead:	CREA-AN
Related Work Package:	WP5
Related Task:	Task 5.1 (R) Laboratory scale manufacturing of fermented shelf-stable sea-fennel preserves at laboratory scale
Author(s)	Dr Valentina Melini (CREA-AN)
Dissemination level	PU
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Summary of Deliverable D5.1 – Pilot Scale Prototypes of Sea Fennel-Based Fermented Preserves	Building on laboratory experiments, the consortium created fermented prototypes - sea fennel beer, kimchi-like preserve, fermented sea fennel sprouts and pickles - demonstrating how this underutilized crop can enrich both Mediterranean traditions and modern food innovation. The sea fennel beer was brewed using both conventional and non-conventional yeast strains, combined with recycled brewers' spent grain and sea fennel extracts. Results showed that incorporating sea fennel enhanced the beer's phenolic content, aromatic complexity, and color intensity, while maintaining good fermentation performance. Volatile compound analysis revealed fruity and floral notes, opening possibilities for a distinctive functional craft beer with Mediterranean identity. The second prototype, a kimchi-style vegetable preserve enriched with sea fennel sprouts, was tested under both spontaneous and starter-driven fermentation. Physicochemical and microbiological monitoring confirmed successful fermentation, with safe acidity levels and the dominance of beneficial lactic acid bacteria. Sensory analysis highlighted a balanced flavor profile, with descriptors such as pungent, garlicky, and vegetal, complemented by the unique herbal character of sea fennel. Fermented sea fennel sprouts in vinegar were produced at laboratory scale using sprouts from a local farm in Italy. Two fermentation batches (November 2022 and July 2023) were prepared with standardized brine and inoculated with selected lactic acid bacteria. Monitoring showed a slight decrease in lactobacilli and mesophilic aerobes by the end of fermentation, while

	Enterobacteriaceae were fully eliminated in Batch 1 and reduced in Batch 2. Yeasts increased steadily, reaching over 5 Log CFU mL^{-1}. These results confirm the feasibility of controlled fermentation of sea fennel as a safe and microbiologically stable product. Fermented sea fennel leaves and sprouts were processed into 24 pickle prototypes using wine and apple vinegar at four acidity levels (0.05%, 0.2%, 0.5%, 0.7%). All samples underwent mild pasteurization (74 °C, 3 min) and were monitored for six months for physicochemical, microbiological, and sensory quality. Microbiological analysis confirmed product safety, with Enterobacteriaceae and major pathogens undetected; limited aerobic and yeast growth was observed at the lowest acidity (0.05%). Sensory evaluation highlighted batch differences in texture stability, with winter-produced samples outperforming summer ones. Moderate acidity (0.5%) yielded the best balance between safety, stability, and sensory quality, enhancing sea fennel's characteristic aroma without excessive acidic flavor.

Versioning and Contribution History

Version	Date	Modified by	Modification reason
v1.0	20/04/2024	Valentina Melini	First version
V2.0	30/04/2024	Valentina Melini	Comments after peer reviewing process

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1. Italian prototypes

Sea fennel edible fresh leaves and sprouts provided by RINCI SrL (P3) were exploited by UNIVPM (P1) for development, production and validation of the following **FERMENTED FOOD LABORATORY-SCALE PROTOTYPES**:

- BEER (addition of extracts produced in WP6 or of fresh sea fennel leaves/flowers during boiling)
- KIMCHII-LIKE PRESERVE (natural and started fermentation of mixtures of vegetables including sea fennel sprouts)
- FERMENTED SEA FENNEL IN VINEGAR

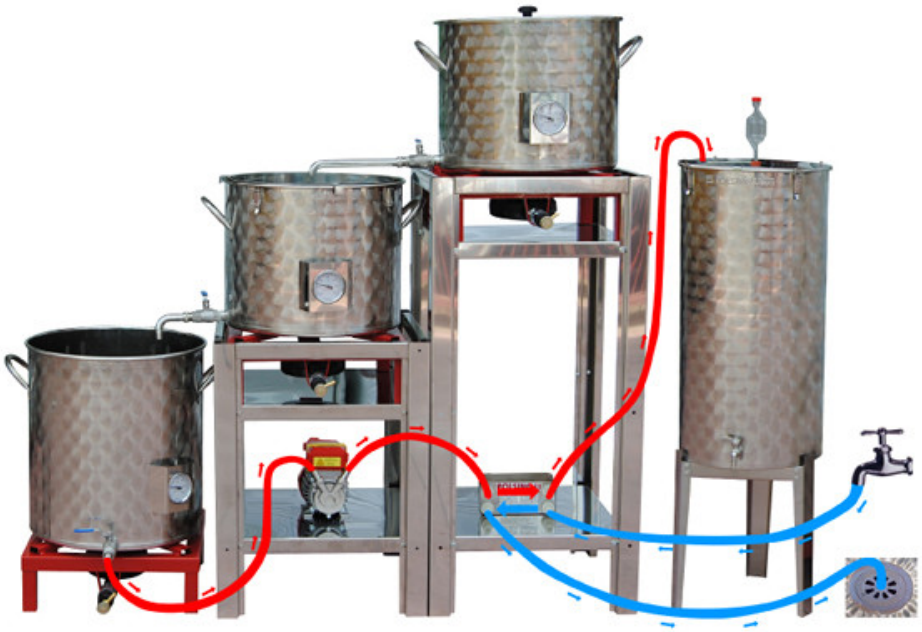
1.1 Sea fennel-based BEER

Material and Methods

Beer formulation

The brewing test was performed at lab scale and at pilot scale.

Lab scale brewing plant (25 L).



Pilot brewing plant (100L).

Yeast starter formulation

The non-conventional yeast strains, coming from the Department of Life and Environmental Sciences (DiSVA) Collection, and the *S. cerevisiae* US-05 (Fermentis, Lesaffre, France) starter strain used as control are reported in the following table. The five yeasts strains were selected taking into account the results of a previous work regarding the analytical and sensory profile of beers (Canonico et al., 2023).The yeast strains used in the trials were cultivated and maintained in YPD agar medium (10 g/L yeast extract, 20 g/L peptone, 20 g/L glucose and

18 g/L agar) at 4 ◦C for short-term storage, while for long-term storage, YPD broth supplemented with 40% (w/v) glycerol at −80 ◦C was used.

Taxonomic classification, strain code and source of isolation of the yeast strains tested and S.cerevisiae commercial strain US-05 (Fermentis, Lesaffre, France).

Brewer's Spent Grain (BSG) and Substrate of Fermentation/Recycled BSG to Produce Worts with Low Sugar Content: BSG +HOP and BSG + SEA FENNEL

The BSG used for the preparation of wort with low sugar content, came from a PILS wort (170 Kg of malt Pils in water at 35 ◦C) added with Cascade hops during the boiling phase. The batch was used to prepare 1000 L of Pils craft beer at the Birra dell' Eremo craft brewery (Assisi, Italy). The wort obtained (1000 L) had the following analytical characteristics: pH 5.5, density 12.3◦P (Plato degree) and 20 IBU (International Bitterness Unit). At the end of the filtration the wort with the characteristics reported above was destined for the craft beer production. At this point, the BSG was further filtered using water at 78 ◦C. Four hundred litres were then collected, and the sugars (glucose, sucrose and maltose) were determined using an enzymatic method (Megazyme, Wicklow, Ireland). Subsequently A total of 15 L of combined wort from exhausted BSG was added with 18 g of Cascade hops and boiled for 1 h. The hopped substrate was divided equally into two batches. In the first batch, it was kept as is and named recycled BSG +HOP, while, in the second, 5 % sea fennel (BSG+SEA FENNEL). The two worts obtained were used separately as substrates for setting up micro-fermentations.

Micro-fermentations

The micro-fermentations were conducted at a temperature of 20 ◦C ± 2 ◦C using 500 mL flasks equipped with Müller valve to allow the escape of CO2, containing 450 mL of wort from recycled wort. The pre-cultures were prepared using 10% malt extract and incubated for 48 h at 20 ◦C ± 2 ◦C. After incubation, the cells were collected by centrifugation (2000 g for 5 min), resuspended in sterile water and the wort by recycled BSG was inoculated with 1×10^6 cells/mL using the Thoma-Zeiss chamber. The inoculated flasks were placed in thermostat at a temperature of 20 ◦C ± 2 ◦C. A control trial without inoculation was also placed in thermostat. The fermentation evolution was monitored recording the decrease in weight due to the loss of CO_2 daily to a constant value. At the end of the resting period at 4 ◦C, the beers underwent refermentation in the bottle by residual and

Species	Strain code	Source of isolation
Lachancea thermotolerans	101	Grapes
Pichia kluyveri	PRMB7	Grapes
Wickerhamomyces anomalus	3003	Backery
Torulaspora delbrueckii	33	Papaya leaves
Saccharomyces cerevisiae	US05	Commercial starter

still viable yeasts, adding 1.5 g/L of sucrose during the bottling phase. The sealed bottles were kept at 18–20 ◦C for about 7–10 days, finally stored at 4 ◦C.

Microbiological and chemical analysis

Viable cell counts were carried out at the start of fermentation process.

Ethanol was measured by gas–liquid chromatographic analysis (Canonico, Zannini, Ciani, & Comitini, 2021). Acetaldehyde, ethyl acetate and the higher alcohols were determined by direct injection of FID -GC (GC-2014; Shimadzu, Kyoto, Japan). The samples were injected into a 2 m × 2 mm i.d. glass column, packed with 80/100 Carbopack C/0.2% Carbowax 1500 (Supelco, Sigma Aldrich, Milan, Italy), with an internal standard of the carrier gas. A Shimadzu gas chromatograph (Kyoto, Japan) equipped with a flame ionization detector was used. The oven temperature ranged from 45 to 160 ◦C. The temperature of the injector and the detector was 220 ◦C. The temperature of the injector and the detector was 220 ◦C as reported by Canonico et al. (2015). The solid-phase microextraction (HS-SPME) method was used to determine the concentration of the volatile compounds. Five mL of beer was placed in a vial containing 1 g NaCl closed with a septum-type cap. HS-SPME was carried out with magnetic stirring for 10 min at 25 ◦C. After this period, the internal standard (3-octanol) (Sigma Aldrich, Milan, Italy) at a concentration of 1.6 mg/L was added, and the sample was heated to 40 ◦C. Divinylbenzene/carboxen/polydimethylsiloxane (DVB/CAR/ PDMS) fibre (Sigma-Aldrich, Milan, Italy) was inserted into the vial headspace for 30 min. The compounds were desorbed by inserting the fibre into a Shimadzu gas chromatograph GC injector for 5 min. A glass capillary column was used: 0.25 µm Supelcowax 10 (length, 60 m; internal diameter, 0.32 mm). The fibre was inserted in the split–splitless mode (Canonico, Comitini, & Ciani, 2015). The compounds were identified and quantified by comparisons with external calibration curves for each compound. Specific enzymatic kits (Megazyme, Wicklow, Ireland) were used to determine the concentrations of glucose sucrose, maltose (kit k-masug, Megazyme, Wicklow, Ireland), ethanol (K-ETOH), lact acid (K-LATE) according to the manufacturer instructions.

Physico-chemical and technological parameters evaluated in the prototypes of beer

Total soluble solids, total acidity, pH, alcoholic grade (% v/v), color (EBC), and bitterness (IBUs) were determined as physico-chemical parameters in control and enriched beers. After 6 months of maturation, the samples from three bottles were analysed in duplicate.

The total soluble solids, expressed as °Brix, and pH were measured by using a digital refractometer at 20°C, and a pH meter, respectively.

Total acidity was measured by titration with 0.1 N NaOH up to pH 8.1, using 1 mL of beer in 25 mL of distilled H2O, and results were expressed as % lactic acid (g lactic acid equivalent per 100 mL).

Alcoholic grade was calculated by using the density data of the beers, which were obtained with a hydrometer before and after fermentation.

Colour EBC and Bitterness (IBUs) were determined according with the Official Methods of the Analytical Division of European Brewery Convention, EBC Method 9.6 and 9.8, respectively, using a spectrophotometer (Varian 5000 UV-Vis NIR) (Abellán et al. 2021).

Liters post-boiling, mashing: time and temperature, mashing-out: time and temperature, primary fermentation step: time and temperature, secondary fermentation: time and temperature

Total Phenolic Content (TPC) determination

The total phenolic content (TPC) of the control (HOP) and sea fennel extract enriched beer (SFB) samples was determined according to the Folin–Ciocalteu method. Briefly, 20 µl of the sample was mixed with Folin reagent, incubated, then Na_2CO_3 solution was added and after 30 min in the dark, absorbance was measured at 750 nm in a UV-Vis spectrophotometer (Onda, UV-31 SCAN, Beijing, China). Each sample was analyzed in triplicate and

the results were expressed as mg gallic acid equivalents (GAE)·g^{-1} of the sample, using a calibration curve of gallic acid.

Volatile compounds by means of SPME-GC-MS

Degassed beer (8 mL) sample and 25 µL internal standard 4-methyl-2-pentanol (534 mg/L) were placed into a 20 mL glass vial for 5 min at 50°C. A HS-SPME fiber (100 µm, DVB/CAR/PDMS, 1 cm, Merck Life Science, Milan, Italy) was exposed for 40 min at 50°C. The fiber was desorbed of volatile compounds in the GC inlet at 250 °C for 5 min. Two columns were used (DBWAX, 60 m × 0.25 mm × 0.25 µm; DB-5MS 30m × 0.25 mm x 0.25 µm) for the untargeted volatile profile of beer for polar and non-polar compounds, respectively. An Agilent 7890 GC equipped with a 5977A MS (New York, USA) was used. The carrier gas helium flowed at the rate of 1 mL/min. For the DBWAX untargeted volatile, the temperature program started at 40 °C, then increased to 120 °C (3 °C/min) and held for 5 min, and finally increased to 230 °C (5 °C/min). For the DB-5MS untargeted volatile analysis, the initial temperature of the oven was 60°C for 1 min, increased to 120°C at 5°C/min rate, held for 3 min and raised to 200°C at a rate of 8°C/min. Finally, the temperature was increased to 250°C at a rate of 10°C/min and held for 1 min. The mass detector was set at 230 °C, and its electron ionization (EI) energy was 70 eV. The mass spectra were obtained in duplicate under full scan acquisition mode with a mass scan range of m/z 33-450 to achieve HS-SPME-GC-MS fingerprints to be used for PCA and PLS-DA elaboration.

References

Canonico, L., Agarbati, A., Comitini, F., & Ciani, M. (2023). Unravelling the potential of non-conventional yeasts and recycled brewers spent grains (BSG) for non-alcoholic and low alcohol beer (NABLAB). *LWT, 190*, 115528. https://doi.org/10.1016/j.lwt.2023.115528

Canonico, L., Zannini, E., Ciani, M., & Comitini, F. (2021). Assessment of non-conventional yeasts with potential probiotic for protein-fortified craft beer production. *LWT, 145*, 111361. https://doi.org/10.1016/j.lwt.2021.111361

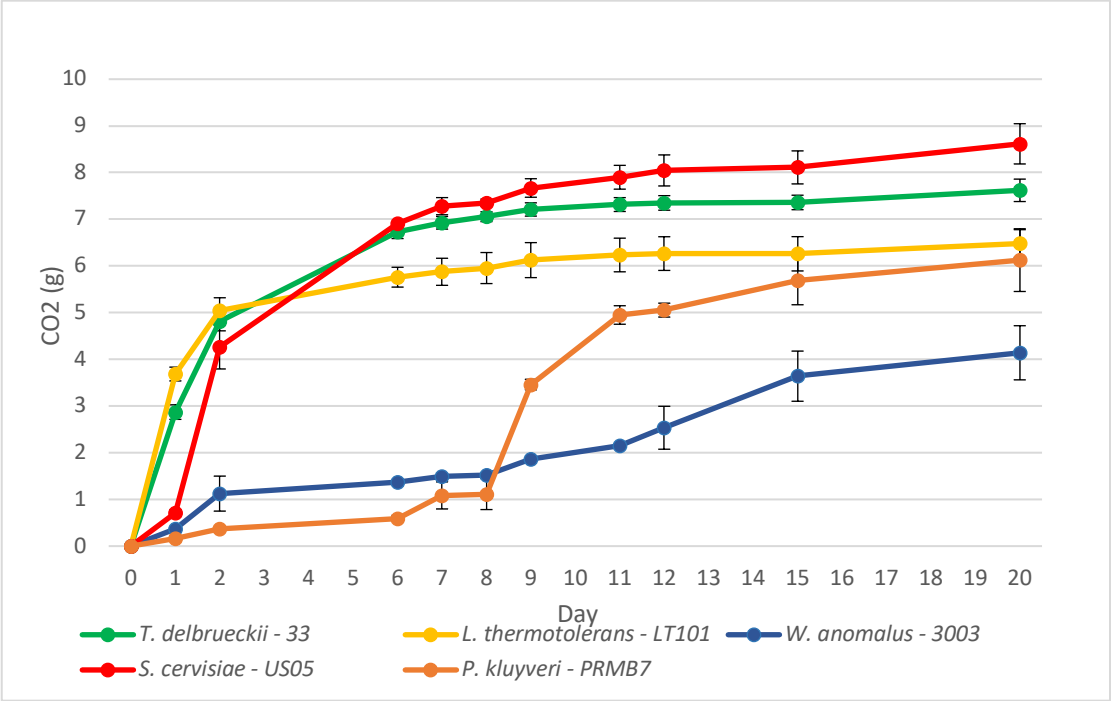
Canonico, L., Comitini, F., & Ciani, M. (2015). Influence of vintage and selected starter on *Torulaspora delbrueckii/Saccharomyces cerevisiae* sequential fermentation. *European Food Research and Technology, 241*, 827–833. https://doi.org/10.1007/s00217-015-2507-x

Results

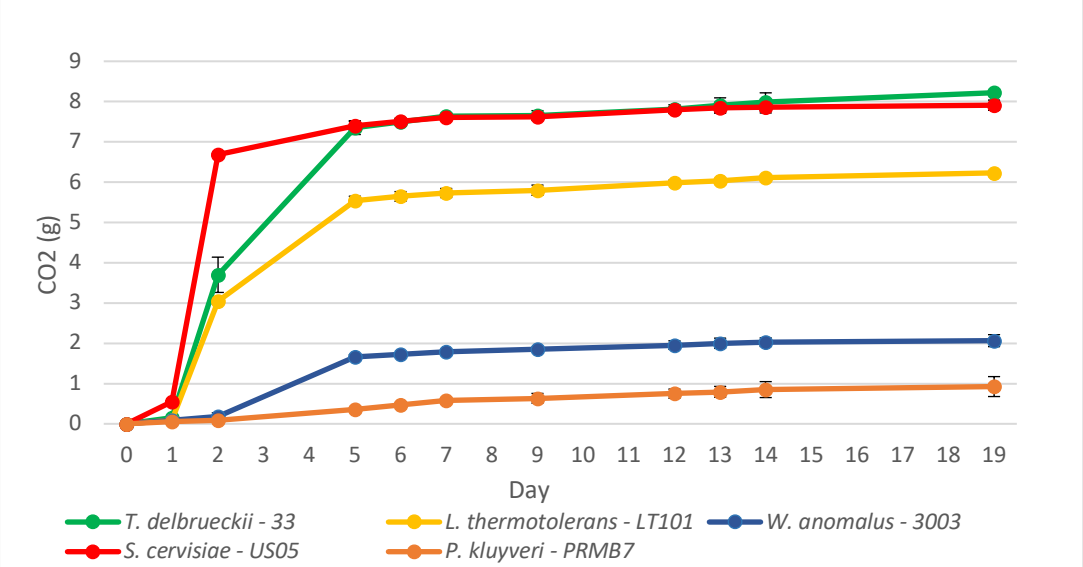
The mashing step started at 62°C (hold 20 min), increased till 68°C in 5 min and hold for 40 min. The mashing out step (78°C) was reached in 10 min and hold for 10 min. Wort was boiled for 60 min with Cascade hop and added of the special ingredient to characterize each different beer sample. After the whirlpool step, wort was filtered and cooled down till 18°C, inoculated with yeast. The fermentation lasted 10 days till the density dropped from around 1.060 till 1.010 g/L. Beer was bottled (750 mL dark glass bottles), added of sugar (2.5 g/L), closed with crown caps, and kept at room temperature for maturation for 6 months.

Fermentation kinetics

The fermentation evolution of the non-conventional yeasts and *S.cerevisiae* commercial strain US-05 on BSG+HOP and BSG+ SEA FENNEL are shown in the following two figures.
The fermentation kinetics of the strain tested on BSG+HOP showed that S. cerevisiae US-05 (starter commerciale) exhibited the highest fermentation evolution (8,61 g di CO_2). A slight reduction in fermentation kinetics was exhibited by *T. delbrueckii 33*, di 7,62 g di CO_2 and *L. thermotolerans LT101* 6.48 g di CO_2.
W. anomalus 3003 and *P. kluyverii PRMB7* exhibited the slower fermentation kinetics. Generally these trends were exhibited by the strain on the BSG+SF.



Fermentation kinetics of the yeasts strain tested on BSG+HOP



Fermentation kinetics of the yeasts strain tested on BSG+ SEA FENNEL

Sugar content analysis

The following table illustrates the results of sugar concentration in the initial substrate.

The results showed an increase maltose consumption in the BSG+Sea Fennel substrate compared to BSG+Hop for *W. anomalus* 3003 and *L. thermotolerans* LT101. Regarding residual sucrose, both substrates exhibited generally similar concentrations, except for *W. anomalus* 3003, which displayed higher residual sucrose levels in BSG+Sea Fennel. Additionally, glucose utilization was generally complete in both prototypes. Finaly, *S. cerevisiae* US05 demonstrated consistent patterns in sugar utilization across both conditions.

Sugar content in the initial substrate before fermentation

Sugar (g/L sample)	BSG+HOP	BSG+SF
Glucose	4,8	5,8
Sucrose	5,3	2,5
Maltose	16,4	17,0

Residual Sugar after fermentation on BSG+HOP

Residual sugar (g/L)	T. delbrueckii 33	L. thermotolerans LT101	W. anomalus 3003	P. kluyveri PRMB7	S. cerevisiae US05
GLUCOSE	0,03 ± 0,00	0,01 ± 0,02	0,20 ±0,23	0,00 ± 0,20	0,02 ± 0,02
SUCROSE	0,05 ± 0,03	0,06 ± 0,07	0,89 ± 0,88	5,13 ± 0,65	0,00 ± 0,14
MALTOSE	1,72 ± 0,12	3,54 ± 2,53	10,11± 0,12	14,13 ± 0,85	0,88 ± 0,02

Residual sugar after fermentation in BSG+ SEA FENNEL

Residual sugar (g/L)	T. delbrueckii 33	L. thermotolerans LT101	W. anomalus 3003	P. kluyveri PRMB7	S. cerevisiae US05
GLUCOSE	0,04 ± 0,02	0,05 ±0,02	0, ±0,92	0,00 ± 0,12	0,00 ± 0,12
SUCROSE	0,00 ± 0,10	0,00± 0,28	4,48 ± 3,03	2,34 ± 3,12	0,03 ± 0,01
MALTOSE	1,36 ±0,39	1,73 ± 0,97	4,32 ± 2,65	13,40 ± 4,70	0,43 ± 0,38

Lactic acid and ethanol content

The addition of sea fennel to the BSG substrate had a minimal impact on both lactic acid and ethanol production. Slight increases in lactic acid and ethanol levels were observed for specific strains such as *T. delbrueckii* 33 and *L. thermotolerans* LT101. Overall, the fermentation outcomes were comparable between BSG+HOP and BSG+Sea Fennel, suggesting that sea fennel has a limited effect on the metabolic activity of the tested yeast strains.

Lactic acid and ethanol content in BSG+HOP

Yeast strains	Lactic acid (g/L)	Ethanol (% v/v)

T. delbrueckii 33	0,00 ± 0,00	0,86 ± 0,01
L. thermotolerans LT101	0,06 ± 0,01	0,75 ± 0,00
W. anomalus 3003	0,00 ± 0,00	0,31 ± 0,00
P. kluyveri PRMB7	0,00 ± 0,00	0,3 ± 0,00
S. cerevisiae US05	0,01 ± 0,00	1,45 ± 0,00

Lactic acid and ethanol content in BSG+ SEA FENNEL

Yeast strains	**Lactic acid** (g/L)	**Ethanol** (% v/v)
T. delbrueckii 33	0,01 ± 0,00	0,93 ± 0,00
L. thermotolerans LT101	0,08 ± 0,01	0,92 ± 0,00
W. anomalus 3003	0,00 ± 0,00	0,3 ± 0,00
P. kluyveri PRMB7	0,01 ± 0,00	0,3 ± 0,00
S. cerevisiae US05	0,00 ± 0,00	1,37 ± 0,00

Alcohol composition

The analysis revealed differences in volatile alcohol compounds between the BSG+HOP and BSG+Sea Fennel substrates. Acetaldehyde contentwas higher in BSG+Sea Fennel for *T. delbrueckii* 33 and *S. cerevisiae* US05, whereas *P. kluyveri* PRMB7 exhibited lower levels compared to BSG+HOP. Ethyl acetate production was generally higher in BSG+Sea Fennel, in *W. anomalus* 3003 *and L. thermotolerans* LT101 showing the highest increases. n-Propanol levels were consistent across substrates for most strains, though *W. anomalus* 3003 produced detectable amounts only in BSG+HOP. Isobutanol and amyl alcohol concentrations were elevated in BSG+Sea Fennel for some strains, such as *T. delbrueckii* 33. However, isoamylic alcohol levels were lower in BSG+Sea Fennel, potentially softening the intensity of fusel alcohol contributions. These variations highlight how the addition of sea fennel may influence the aromatic and flavor profile of the beer.

Alcohol composition in BSG+HOP

Alcoli (ppm)	*T. delbrueckii 33*	*L. thermotolerans LT101*	*W. anomalus 3003*	*P. kluyveri PRMB7*	*S. cerevisiae US05*
Acetaldehyde	0,00±0,00^{c}	0,00±0,00^{c}	5,46±2,04^{b}	8,5±0,68^{a}	0,00±0,00^{c}
Ethyl acetate	121,26±1,36^{a}	114,40±0,62^{b}	84,70±3,96^{c}	5,4±1,38^{e}	58,48±2,91^{d}
n-propanol	10,73±0,18^{b}	0,00±0,00^{c}	12,64±0,12^{b}	0,00±0,00^{c}	26,16±0,35^{a}
Isobutanol	5,69±2,42^{a}	0,00±0,00^{b}	0,00±0,00^{b}	0,00±0,00^{b}	0,00±0,00^{b}
Amyl alcohol	1,361±0.2^{b}	44,81±0,71^{a}	1,33±0,04^{b}	0,00±0,00^{c}	0,00±0,00^{c}
Isoamylic alcohol	20,03±0,49^{a}	0,00±0,00^{c}	15,27±0,03^{b}	0,00±0,00^{c}	20,40±0,56^{a}

Alcohol composition in BSG+SEA FENNEL

Alcoli (ppm)	*T. delbrueckii 33*	*L. thermotolerans LT101*	*W. anomalus 3003*	*P. kluyveri PRMB7*	*S. cerevisiae US05*
Acetaldehyde	5,93±0,21^{a}	0,00±0,00^{e}	0,86±0,02^{d}	2,06±0,04^{c}	3,30±0,14^{b}
Ethyl acetate	235,50±5,45^{c}	317,87±0,60^{b}	539,71±4,10^{a}	81,00±0,26^{d}	48,42±24,78^{e}
n-propanol	10,41±0,59^{b}	10,12±0,30^{b}	0,00±0,00^{b}	10,19±1,30^{b}	19,88±1,10^{a}
Isobutanol	30,47±4,65 ^{a}	9,78±0,57^{b}	0,00±0,00^{d}	0,00±0,00^{d}	5,68±0,37^{c}
Amyl alcohol	18,53±3,56^{a}	0,00±0,00^{b}	0,00±0,00^{b}	0,00±0,00^{b}	16,40±0,65^{a}
Isoamylic alcohol	2,04±0,88^{a}	0,00±0,00^{b}	0,00±0,00^{b}	0,00±0,00^{b}	0,00±0,00^{b}

Volatile compounds

The inclusion of sea fennel in the BSG substrate led a general increases in some ester compounds, such as isoamyl acetate andethyl hexanoate, which may enhance fruity and floral aroma profiles. However, β-phenyl ethanol production decreased in some strains. Overall, the use of sea fennel as a substrate modifier appears to influence the volatile composition variably across different yeast strains, with notable enhancements in specific aroma-active compounds.

Volatile compounds composition on BSG+HOP

Volatile compounds (mg/L)	*T. delbrueckii* 33	*L. thermotolerans* LT101	*W. anomalus* 3003	*P. kluyveri* PRMB7	*S. cerevisiae* US05
Isoamyl acetate	10,92±0,18^{a}	7,46±2,98^{a}	0,63±0,03^{b}	9,67±2,19^{a}	0,47±0,00^{b}
Ethyl hexanoate	0,03±0,00^{b}	0,00±0,00^{c}	0,08±0,01^{a}	0,04±0,01^{b}	0,04±0,01^{b}
hexanol	0,01±0,00^{c}	0,06±0,00^{a}	0,01±0,00^{c}	0,01±0,00^{c}	0,04±0,00^{b}
Ethyl octanoate	0,02±0,01^{a}	0,01±0,00^{a}	0,00±0,00^{b}	0,00±0,00^{b}	0,00±0,00^{b}
Linalol	0,01±0,00^{b}	0,05±0,01^{a}	0,00±0,00^{b}	0,00±0,00^{b}	0,01±0,00^{b}
diethylsuccinate	0,01±0,00^{b}	0,02±0,00^{b}	0,00±0,00^{c}	0,03±0,00^{a}	0,00±0,00^{c}
α - Terpineol	0,02±0,00^{ab}	0,04±0,01^{a}	0,01±0,00^{b}	0,04±0,01^{a}	0,01±0,00^{b}
Citronellol	0,09±0,01^{a}	0,03±0,00^{b}	0,08±0,00^{a}	0,10±0,03^{a}	0,04±0,00^{b}
phenyl ethyl acetate	0,00±0,00^{b}	0,02±0,00^{a}	0,03±0,01^{a}	0,00±0,00^{b}	0,01±0,01^{a}
Nerol	0,00±0,00^{b}	0,01±0,00^{a}	0,00±0,00^{b}	0,01±0,00^{a}	0,01±0,00^{a}
Geraniol	0,00±0,00^{b}	0,01±0,00^{b}	0.04±0,01^{a}	0,00±0,00^{b}	0,01±0,00^{b}
β-phenyl ethanol	4,14±0,10^{d}	7,30±1,00^{b}	0,00±0,00^{e}	3,30±0,12^{c}	13,96±0,90^{a}

The main volatile compounds on BSG+SEA FENNEL

Volatile compounds (mg/L)	T. delbrueckii 33	L. thermotolerans LT101	W. anomalus 3003	P. kluyveri PRMB7	S. cerevisiae US05
Isoamyl acetate	11,35±0,28^{b}	19,88±0,90^{a}	8,74±0,17^{c}	11,76±1,09^{b}	1,25±0,07^{d}
Ethyl hexanoate	0,08±0,00^{c}	0,54±0,01^{a}	0,12b±0,00	0,04±0,00^{c}	0,06±0,00^{c}
hexanol	0,00±0,00	0,06±0,00 ^{a}	0,00±0,00	0,01c±0,00	0,02±0,00 ^{b}

Ethyl octanoate	0,01±0,00^{a}	0,01±0,00^{a}	0,01±0,00^{a}	0,00±0,00	0,01±0,00^{a}
Linalol	0,02±0,00 ^{b}	0,05±0,01^{a}	0,01±0,00^{c}	0,04±0,01^{a}	0,01±0,00^{c}
diethylsuccinate	0,01±0,00^{b}	0,02±0,00^{a}	0,02±0,00^{a}	0,02±0,00^{a}	0,00±0,00^{c}
α - Terpineol	0,05±0,00^{b}	0,01±0,00^{d}	0,09±0,00^{a}	0,03±0,02^{c}	0,00±0,00
Citronellol	0,04±0,00^{bc}	0,01±0,02^{c}	0,01±0,00^{c}	0,33±0,01^{a}	0,10±0,03^{b}
phenyl ethyl acetate	0,00±0,00^{c}	0,00±0,00^{c}	0,03±0,01^{b}	0,00±0,00^{c}	0,13±0,01^{a}
Nerol	0,03±0,00^{a}	0,00±0,00^{c}	0,00±0,00^{c}	0,02±0,00^{b}	0,00±0,00^{c}
Geraniol	0,00±0,00	0,00±0,00	0,00±0,00	0,01±0,00^{b}	0,02±0,01^{a}
β-phenyl ethanol	3,45±0,23^{b}	0,50±0,00^{d}	0,77±0,00^{c}	4,45±0,75^{b}	8,82±0,07^{a}

Color and pH

The Color and pH of control (HOP) and sea fennel extract enriched beer (SFB)

	HOP			SFB		
Before fermentation	pH	Color (EBC)	Bitter (IBUs)	pH	Color (EBC)	Bitter (IBUs)
TREB	5.65±0.04^{c}	15.12±0.12^{c}	11.92±0.62^{a}	5.45±0.01^{c}	38.62±0.12^{c}	20.35±1.1^{d}
After fermentation						
33	4.26±0.03^{a}	12.37±0.12^{b}	20.1±0.3^{c}	4.27±0.04^{a}	45.50±0.75^{c}	18.62±0.47^{cd}
3003	4.28±0.04^{a}	12.50±0.25^{b}	24.22±0.77^{d}	4.69±0.09^{b}	31.87±0.12^{b}	16.92±0.52^{bc}
LT101	4.25±0.02^{a}	11.75±1.00^{a}	17.67±0.17^{b}	4.71±0.03^{b}	27.00±0.01^{a}	13.77±0.22^{a}
PK	4.33±0.04^{a}	11.75±0.00^{a}	18.1±0.55^{b}	4.99±0.06^{b}	38.12±0.62^{c}	15.77±0.42^{ab}
US05	4.62±0.05^{b}	19.12±1.87^{d}	24.42±0.02^{d}	4.82±0.00^{b}	31.25±0.75^{b}	16.6±1.25^{bc}

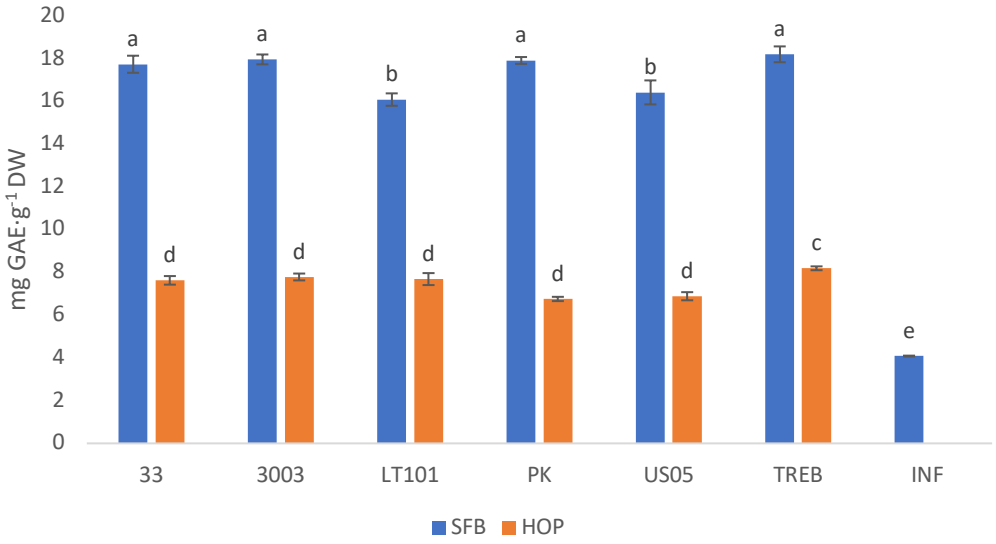
Values are presented as mean of three replicates ± SD. Different letters in each column indicate statistical differences ($p < 0.05$).
EBC, European Brewing Convention
IBUs, International Bitterness Units

The physiochemical results of HOP and SFB beer including pH, color and bitterness across various yeast strains are shown in Table 1. Prior to fermentation, the pH values of the sea fennel extract-enriched beer (SFB) and the control beer

(HOP) were higher than those following fermentation. For instance, depending on the yeast strain, SFB's pH varied from 4.27 to 4.99. These values were greater than the 4.25 to 4.62 ranges found in HOP beer. The impact of sea fennel extract may be the cause of this discrepancy.

Higher EBC values were seen in SFB compared to HOP beers, especially where 33 has the highest color intensity (45.50±0.75 EBC), suggesting that the addition of sea fennel extract produced a more intense or dark beer color. All samples showed the same color increase, indicating that the extract had a consistent impact. The bitterness was constantly higher in the control beer condition with maximum value of 24.42±0.02 IBUs, except for TREB, which has higher IBUs under SFB (20.35±1.1 IBUs).

Total phenolic content



TPC (total phenolic content) in control beer (HOP) and sea fennel extract enriched beer (SFB)
Data are expressed as mean ± standard deviation (n= 3) mg gallic acid equivalents/g dw (dry weight).
Different letters indicate statistical differences ($p < 0.05$).

The total phenolic content of the SFB and the HOP across several yeast strains is shown in Figure 1. When compared to HOP beers, SFB consistently exhibit a significantly higher total phenolic content, ranging from 16.06 to 17.94 mg GAE·g−1 DW, with 3003 showing the highest value. In contrast, the range of HOP samples is from 6.74 to 7.77 mg GAE·g^{-1} DW. The TREB sample, which was taken prior to fermentation, had a slightly higher total phenolic content under both HOP and SFB conditions than the other beer samples. In comparison to the other samples, the sea fennel infusion's (INF) total phenolic content was significantly lower, at just 4.08 mg GAE·g^{-1} DW.

References

Abellán, Á.; Domínguez-Perles, R.; Giménez, M.J.; Zapata, P.J.; Valero, D.; García-Viguera, C. The Development of a Broccoli Supplemented Beer Allows Obtaining a Valuable Dietary Source of Sulforaphane. Food Biosci. 2021, 39, doi:10.1016/j.fbio.2020.100814.

Fanesi, B.; Ismaiel, L.; Nartea, A.; Orhotohwo, O.L.; Kuhalskaya, A.; Pacetti, D.; Lucci, P.; Falcone, P.M. Bioactives and Technological Quality of Functional Biscuits Containing Flour and Liquid Extracts from Broccoli By-Products. Antioxidants 2023, 12, doi:10.3390/ANTIOX12122115/S1.

1.2 Kimchi-like preserve

Material and Methods

Microbial starter formulation

The starter culture of lactic acid bacteria used for kimchi product was formulated and selected according to their technological traits. The same strains have been applied in the fermentation of sea fennel sprouts in a brine salt solution (Maoloni et al., 2021). The starter culture was composed of 4 different strains ascribed to: *Lactiplantibacillus plantarum* (strain PB257), *Leuconostoc pseudomesenteroides* (strain PB288), *Pediococcus pentosaceus* (strain FF78), and *Weissella confusa* (strain PB 321). This culture belongs to the Culture Collection of the Department of Agricultural, Food, and Environmental Sciences (D3A, Università Politecnica delle Marche), where 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 sub cultured in MRS broth (VWR) at 30°C for 24 h, prior to their use.

Kimchi production

Started and non-started (control) Kimchi prototypes were prepared. The sea fennel sprouts used for Kimchi prototypes was supplied by the local farm of Rinci company (S.r.l, Castefidardo, Ancona, Italy). All the ingredients including sea fennel sprouts used for Kimchi production are reported in the table below.

Ingredients used for Kimchi production.

	% ingredient	Ingredient (g)
Chinese cabbage	70.53%	3725.34
Onion	4.37%	230.62
Garlic	0.50%	26.61
Ginger	0.50%	26.61
Sea fennel	7.05%	372.53
Red pepper	0.17%	8.87
Paprika	1.85%	97.57
Sugar	0.84%	44.35
Salt	0.76%	39.91
Water	13.43%	709.59

The production was performed according to the steps described as followed: 10 kg of Chinese cabbage (Brassica rapa subsp. pekinensis) were cleansed, cut in 4 parts and then in 4 cm width strips, resulting in a total weight of 9.3 kg.

Chinese cabbage (Brassica rapa subsp. pekinensis) ready to be processed for Kimchi production.

The vegetable has been washed in water and soaked in a 10.9 % NaCl brine (9.3 kg Chinese cabbage + 40.6 kg water + 5 kg NaCl) for about 14 hours. The cabbage has been washed by immersion in 100 L water, performing two rinses. This process resulted in the obtaining of 7.5 kg cabbage to be used for fermentation. The sauce was prepared using the ingredients reported in Table1.

The Chinese cabbage was divided in two equal portions (3725 g each), and one of them was mixed with a sauce inoculated with the starters mentioned previously, to reach a final concentration of 7 Log CFU/g of microbial load. The other portion was added with non-inoculated sauce and was used as a control. For each Kimchi prototype, started and non-started, three replicates were produced where each one consisted of one glass jar filled with total weight of 1Kg kimchi product and transported to the laboratory where the fermentation was conducted at 5 ± 1°C for 26 days (Figure 2).

Kimchi prototype prepared with sea fennel sprouts.

Physical-chemical analyses

pH values were measured using pH meter equipped with an H2031 solid electrode (Hanna Instruments, Padova Italy) directly immersed in the Kimchi product matrix in aseptic conditions after inoculation (day 0) and during the fermentation process up until the end of the monitoring period corresponding to 2, 5, 7, 9, 12, 14, 16, 19, 22 and 26 days. For each sample, pH measurements were carried in triplicate for each replicate and values expressed as mean ± standard deviation.

Titratable acidity (TA) was assessed by aliquoting 10 g of each sample and blending with 90mL of distilled water. The resulting mixture was titrated with 0.1 NaOH and the results expressed as % of lactic acid. TA measurements were performed in duplicate for each replicate and the results reported as mean ± standard deviation.

The quantification of organic acids (lactic acetic acid) was performed as previously described by Maoloni et al. (2021). The determination involved processing deproteinized and decolored Kimchi samples. For the deproteinization, a multi-step process was followed: (i) Carrez I solution was prepared by dissolving 3.6g of potassium hexacyanoferrate (II) {K4[Fe(CN)6] × 3H2O} from Sigma Aldrich, Milan, Italy and (iii) sodium hydroxide solution (NaOH, 100 mM), prepared by dissolving 4 g of NaOH in 1 L of distilled water. Then the samples were decolored using 2% (w v^{-1}) polyvinylpolypyrrolidone.

The concentrations of acetic acid and lactic acid in the Kimchi samples were quantified respectively using the Acetic Acid (Acetate Kinase Rapid Manual Format) Assay kit and the D-/L-Lactic Acid (D-/L-Lactate) (Rapid) Assay kit used both sourced from megazyme, Bray, Ireland.

Microbial enumeration

Microbiological analyses were performed by aliquoting 10g of each replicate and adding 90mL sterile 0.1% (w v-1) peptone water. The suspension was then homogenized for 5min at 230rpm in a stomacher machine (400 Circulator, International PBI, Milan, Italy). Enumeration was performed by preparing tenfold serial dilutions for: (i) mesophilic aerobic bacteria on Plate Count Agar (PCA) (VWR International Srl, Milan, Italy), by incubating at 30°C for 48 h; (ii) presumptive mesophilic lactobacilli 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) presumptive 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 (RB) (VWR), by incubating at 25°C for 5 days; (v) Enterobacteriaceae on Violet Red Bile Agar (VRBGA) (VWR), by incubating at 37°C for 24 h, and (vi) Pseudomonadaceae on Pseudomonas Agar Base (PAB) (VWR) supplemented with Cetrimide-Fucidin-Cephalosporin (CFC) selective supplement incubated at 30°C for 24-48h. The results of viable counting were expressed as the mean Log colony forming units (CFU) g^{-1} of three replicates ± standard deviation.

Aliquots of Kimchi were aseptically collected from each replicate of each sample 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.

Statistical analysis

To assess statistical differences within Kimchi samples, the Tukey-Kramer's Honest Significant Difference (HSD) test (level of significance 0.05) was used by one-way analysis of variance (ANOVA). Tests were performed through JMP v11.0.0 software (SAS Institute Inc., Cary, NC).

Isolation and identification of lactic acid bacteria in control Kimchi

Isolation was performed on non-started Kimchi to identify and characterize the strains that grew during fermentation period in this prototype. Aliquots (10 g) of each replicate were serially diluted in 90 mL of sterile saline peptone water (0.9% NaCl, 0.1% peptone, pH 7.0), and 100 µL of each dilution was streaked on MRS agar under anaerobiosis to count lactobacilli. For each replicate, colonies were selected based on colony morphology. The representative colonies that were selected, corresponding to about 10% of the colonies counted on MRS plates seeded with the highest sample dilution. For each

isolate, the cell morphology was examined using a light microscope under oil-immersion (100×). Bacterial isolates were then stored at −80 °C in a mixture of glycerol and MRS (1:1).

DNA extraction was performed to the total isolates of non-started Kimchi that were previously cryopreserved isolates then first cultured on suitable media (MRS) agar. The colonies were suspended in 300 µL of sterile water; the suspension underwent DNA extraction using the method proposed by Hynes et al. (1992) with some modifications suggested and described by Osimani et al. (2015). In fact, after centrifugation of the isolate's suspensions, the cell pellets were resuspended in 1 mL of STE buffer [10 mM Tris–HCl pH 8.0, 100 mM NaCl, 1 mM EDTA pH 8.0,20% sucrose (w/v)] containing 25 mg mL^{-1} of lysozyme. After incubation at 37°C for 3 h, samples were centrifuged at 14,000g for 3 min, and the pellets suspended in 1 mL of lysis buffer consisting of 50 mM KCl, 10 mM Tris–HCl pH 8.0, 0.45% Tween 20 (w/v) 0.45% Triton X (w/v) supplemented with 100 µg mL^{-1} proteinase K. After incubation at 60°C for 3 h, samples were heated at 95°C for 10 min. The DNA quantity and purity were assessed by optical readings at 260, 280 and 234 nm, respectively, using a UV-Vis Shimadzu UV-1800 spectrophotometer (Shimadzu Corporation, Kyoto, Japan).

As previously described by Cardinali et al. (2024) the lactic acid bacteria isolates underwent molecular identification through sequencing the 16S rRNA gene, using universal eubacterial primers P27f (5'-GAG AGT TTG ATC CTG GCT CAG-3') and P1495r (5'-CTA CGG CTA CCT TGT TAC GA-3'). PCR amplification was performed with 100 ng of template DNA in a 50 µL reaction mixture containing 2 U of Taq DNA polymerase (Euroclone, Pero, Italy), 1× reaction buffer, 2.5 mM MgCl2, 0.2 mM dNTPs, and 0.2 µM of each primer. The amplified products were then purified and sequenced by Genewiz (Takaley, UK). The obtained sequences were analyzed using the Basic Local Alignment Search Tool (BLAST) to identify similarities with 16S rRNA sequences of type strains available in the GenBank database (http://www.ncbi.nlm.nih.gov/). Finally, the sequences corresponding to the lactic acid bacteria isolates were deposited in GenBank to receive accession numbers.

RNA extraction, cDNA synthesis, and metataxonomic analysis

Aliquots of 1.5 mL of each kimchi homogenates (dilution 10^{-1}) were centrifuged at 14,000 rpm for 10 min (Centrifuge 5420, Eppendorf, Hamburg, Germany). The supernatant was carefully removed to obtain cell pellets. For each sample, the pellet was preserved in RNA later® Stabilization Solution (Ambion, Foster City, CA, USA) and stored at −80 °C for subsequent RNA extraction. Total microbial RNA was extracted using the Quick-RNA™ Miniprep Kit (Zymo Research, Irvine, CA, USA) according to the manufacturer's protocol. The extracted RNAs were checked for the quantity, purity and the absence of DNA contamination. Then, SensiFAST cDNA Synthesis Kit (Meridian Bioscience Inc., Cincinnati, Ohio, USA) was used for the synthesis of the cDNA from 10 µL of RNA per sample, following the manufacturer's instructions.
The bacterial community was analyzed by amplifying the V3–V4 hypervariable region of the 16S rRNA gene using primers and procedure described by Klindworth et al. (2013). The fungal population was studied by the amplification of the D1 domain of the 26S rRNA gene according to Mota-Gutierrez et al. (2018). PCR amplicons were purified following the Illumina metagenomic pipeline (Illumina Inc., San Diego, CA, United States). Pair-end sequencing (2X250bp) was performed with a MiSeq platform (Illumina Inc., San Diego, CA, United States) using V2 chemistry according to the manufacturer's instructions.
Raw reads were analyzed by using QIIME2 software; in detail, primers and adapters were first trimmed by using Cutadapter, and then quality filtered using the DADA2 algorithm. Amplicon sequence variants (ASVs) generated through DADA2 were used for taxonomic assignment against the Greengenes database for bacteria, and a manually built database for fungi. When the taxonomy assignment was not able to reach species level, the family or genus name was displayed.
The sequence data were processed and analyzed using Microbiome Analyst (Chong et al., 2020) to assess microbial diversity indices, specifically the Shannon index for alpha diversity and the Bray-Curtis dissimilarity index for beta diversity, following rarefaction to the minimum read depth across all samples. Alpha diversity differences among samples were evaluated using the Kruskal-Wallis test applied to the Shannon index. Beta diversity was visualized through Principal

Coordinate Analysis (PCoA) based on the Bray-Curtis dissimilarity index, with significant differences between groups determined using Permutational Multivariate Analysis of Variance (PERMANOVA) ($p < 0.05$).
ASV tables were further analyzed to identify taxa with statistically significant differences among samples. This was achieved using one-way ANOVA and Kruskal-Wallis test ($p < 0.05$). The results were visualized using box plots, specifically highlighting the taxa with statistically significant differences. All statistical analyses and visualizations were performed within the R environment (version 4.4.3).

Determination of volatile compounds via Headspace/Solid Phase MicroExtraction–Gas Chromatography/MassSpectrometry (HS/SPME-GC/MS)

Ten grams of each homogenized sample were treated with a solution of calcium chloride dihydrate (87 g in 100 mL deionized water) and homogenized for 1 minute using an Ultra Turrax. The resulting mixture was then centrifuged at 2,000 rpm for 15 min at 5°C, and the supernatant was filtered through a Whatman No. 41 filter. The isolation of leaf volatiles was performed using the Headspace Solid Phase Microextraction (HS-SPME) technique on the obtained filtrate. Five mL of the filtrate were placed in a 15 mL vial with a magnetic stir bar for SPME, which was then sealed with a PTFE/silicone septum. The vial was immersed in a water bath maintained at 40 °C. The HS-SPME extraction was conducted by exposing a 2-cm 50/30 µm DVB/CAR/PDMS fiber to the headspace of the filtrate for 60 min, at 40 °C under stirring (400 rpm). After the extraction, the fiber was immediately inserted into the GC split-splitless injection port for desorption, and the GC analysis was initiated. The same fiber was used for all analyses.
GC/MS analyses were performed using an Agilent 6890 GC 5973N MS system equipped with a quadrupole mass filter for mass spectrometric detection (Agilent Technologies, Palo Alto, CA). Desorption of the extracted volatiles from the fiber was carried out for 3 minutes in the GC injector, operating at 240 °C in splitless mode. Following desorption, the fiber was kept in the injector for an additional 5 minutes under purge mode (75 mL min^{-1} purge flow) to remove any residual substances adsorbed on the fiber and minimize carry-over effects. A 0.75 mm liner suitable for SPME analyses was installed in the injector. GC separation was achieved using a DB-Wax column (0.25 mm i.d. × 60 m, 0.5 µm film thickness) from Agilent Technologies. The GC operating conditions were as follows: inlet temperature set to 240 °C; oven temperature programmed from 40 °C (held for 10 min) to 235 °C (held for 7 min) at a rate of 4 °C min^{-1}, with a total run time of 65.7 min. The carrier gas flow rate was set at 2.0 mL min^{-1}, corresponding to a linear velocity of 36.3 cm/s. The transfer line temperature was 240 °C. The MS detector operated in electronic ionization mode at 70 eV, with source and quadrupole temperatures set at 230 and 150 °C, respectively. Detection was performed in full scan mode, covering the mass range 33-300 amu. Compound identification was based on the comparison of mass spectra and linear retention indices (LRI) from chromatograms of kimchi samples with those of authentic standards when available. In the absence of authentic standards, tentative identification was achieved by comparison with data from the NIST/EPA/NIH Mass Spectra Library or relevant literature. LRI were determined for each column using a series of linear alkanes (C7-C30) injected under the same chromatographic conditions. For semi-quantitative determination of volatiles, duplicate extractions of the same filtrate were performed, and GC separation was repeated. The levels of VOCs were estimated based on the area of the chromatographic peaks (Fu et al., 2013).

Sensory analysis

The sensory analysis was performed using the method proposed by Maoloni et al., 2022 with some modifications.

This was carried out at the end of fermentation by a panel consisting of 8 non-smokers, made up of 5 females and 3 males aged between 25 and 45. Panelists were preliminarily trained to describe the attributes of Kimchi and sea fennel. A discussion was planned by the panel to find out the most appropriate sensory attributes of Kimchi together with sea fennel. Regarding the evaluation of the prototypes started and non-started Kimchi consisting of two samples, these were presented to the panelists at room temperature and coded with random numbers. The sensory test was divided into sessions, in which each panelist evaluated 2 samples one at each time, working in individual booths and equipped with still bottled water and crackers biscuits to cleanse the olfactory palate between and after the evaluations.

The two-prototype started, and non-started Kimchi were evaluated by the trained panelists for (i) six olfactory descriptors being fermented, garlic, pungent, chilly, vegetable and sea fennel, (ii) six aromas' descriptors being fermented, garlic, spicy, chilly, vegetable and sea fennel, (iii) four flavor descriptors being acidity, bitterness, salty, and sweet, (iv) three textural descriptors being hardness, fibrousness, and crunchiness, and (v) global acceptance. Aliquots (10g) of each sample per panelist were placed in white plastic cups, blindly labelled with numbers. For each descriptor, the panelists were asked to assign a score ranging from 1 to 9 where 1 expresses the lowest and 9 the highest intensity. They were also invited to express their degree of liking with a 9-point hedonic scale, where 1 presents the lowest and 9 the highest degree of liking (Peryam and Pilgrim, 1957).

Results

Physical-chemical analysis

The results of pH and total titratable acidity of started and non-started Kimchi are shown in the figures below. The two analyzed Kimchi had similar trends, although both samples had a faster decrease in pH and oppositely an increase of TTA. In fact, at day 0, the pH values were 4.87± 0.08 and 4.92 ± 0.08 for non-started and started Kimchi, respectively. A drop in pH is observed at day 12 for started kimchi whereas a slight increase is reported for non-started Kimchi with values of 5.16 ± 0.17 and 4.4 ± 0.13, respectively. The drop of pH during the fermentation period is mostly reported between day 12 and day 19 when started Kimchi had a fast decrease whereas non-started Kimchi had a progressive decrease. Instead on day 26 the results are similar, the values reported are 3.97 ± 0.01 and 3.86 ± 0.04. The decrease of pH is accompanied by an increase of TTA similarly for started and non-started samples from day 0 to day 26. At the end of fermentation on day 26, TTA reached 0.30 ± 0.03 for non-started Kimchi while for started Kimchi the value reported is 0.50 ± 0.04.

Results of pH measurements of started and naturally fermented (control) kimchi during fermentation.

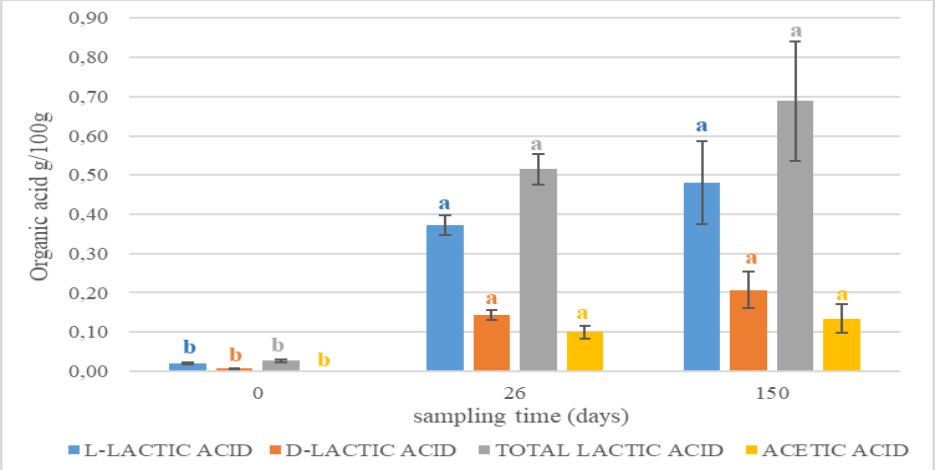
Sampling time (t_{day})	**Prototypes**	
	started kimchi	control kimchi
t_0	$4.90 \pm 0.08^{b,A}$	$4.87 \pm 0.08^{c,A}$
t_2	$5.20 \pm 0.07^{a,A}$	$5.30 \pm 0.17^{ab,A}$
t_5	$5.30 \pm 0.07^{a,B}$	$5.51 \pm 0.13^{a,A}$
t_7	$4.90 \pm 0.10^{b,B}$	$5.46 \pm 0.13^{ab,A}$
t_9	$4.70 \pm 0.15^{bc,B}$	$5.49 \pm 0.07^{ab,A}$
t_{12}	$4.40 \pm 0.13^{cd,B}$	$5.16 \pm 0.17^{bc,A}$
t_{14}	$4.20 \pm 0.03^{d,B}$	$4.97 \pm 0.08^{c,A}$
t_{16}	$4.10 \pm 0.19^{def,B}$	$4.57 \pm 0.08^{d,A}$
t_{19}	$4.00 \pm 0.05^{ef,B}$	$4.19 \pm 0.08^{e,A}$
t_{22}	$3.90 \pm 0.02^{f,B}$	$3.99 \pm 0.01^{e,A}$
t_{26}	$3.90 \pm 0.02^{f,B}$	$3.97 \pm 0.01^{e,A}$

Values are expressed as means ± standard deviations. For each sample, overall means with different superscript letters within the same column are significantly different ($p < 0.05$). For each sampling time, overall means with different small letters within the same row are significantly different ($p < 0.05$).

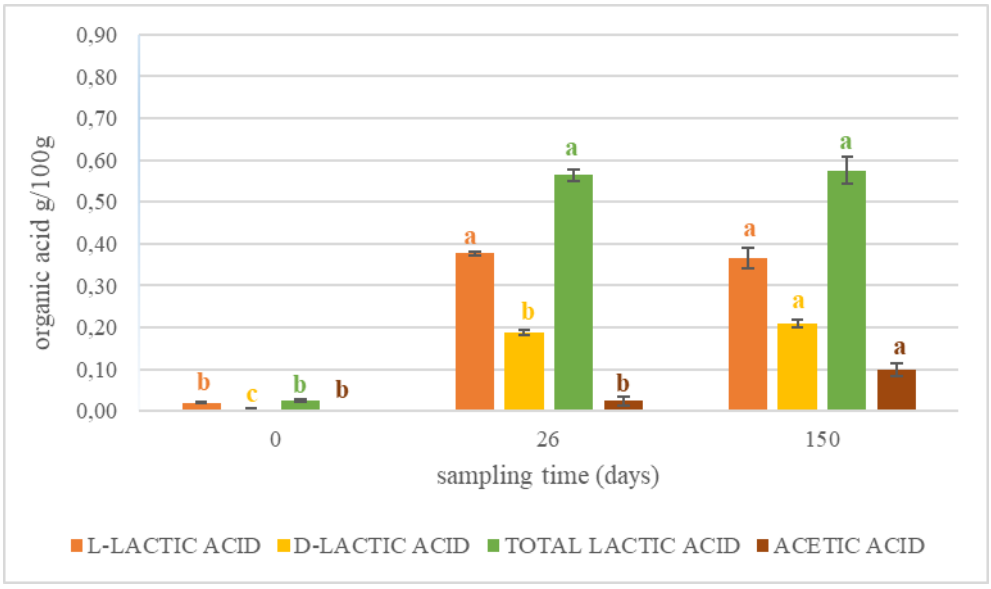
Results of titratable acidity (TA) measurements of started and naturally fermented (control) kimchi during fermentation.

Sampling time (t_{day})	Prototypes	
	started kimchi	control kimchi
t_0	$0.09 \pm 0.01^{b,A}$	$0.09 \pm 0.01^{b,A}$
t_{26}	$0.53 \pm 0.05^{a,A}$	$0.34 \pm 0.03^{a,B}$

Results are expressed as % of lactic acid equivalent. For each sample, overall means with different superscript letters within the same column are significantly different ($p < 0.05$). For each sampling time, overall means with different small letters within the same row are significantly different ($p < 0.05$).



Results of the organic acids quantification of the laboratory scale prototype of started Kimchi made with sea fennel at the beginning, the end of fermentation and after 105 days from the start of fermentation (FK).The results are expressed as mean of three biological replicates ± standard deviation.



Results of the organic acids quantification of the laboratory scale prototype of non-started Kimchi made with sea fennel at the beginning, the end of fermentation and after 105 days from the start of fermentation (CK).
The results are expressed as mean of three biological replicates ± standard deviation.

The results of the organic acids quantification are presented in figures 5 and 6. In details, the total lactic acid content in both started (FK) and non-started (CK) Kimchi increased during fermentation process. For CK samples, values ranged from 0.02 ± 0.00 to 0.56 ± 0.01 g/100g, while for FK, they ranged from 0.03 ± 0.00 to 0.52 ± 0.04 g/100 g. However, after 150 days, the total lactic acid concentration only slightly increased in started Kimchi, reaching 0.69 ± 0.15 g/100g. In almost all samples, the isomer L-lactic acid was more abundant than D-lactic acid.

In contrast, regarding acetic acid, by the end of fermentation, the concentration in started Kimchi was higher than in non-started Kimchi, with values of 0.10 ± 0.02 and 0.02 ± 0.01 g/100g, respectively. However, after 150 days, the acetic acid concentration in non-started Kimchi significantly increased reaching 0.10 ± 0.02 g/100g, whereas it only slightly increased in started Kimchi to 0.13 ± 0.04 g/100g.

In conclusion, both prototypes exhibited a higher yield of lactic acid compared to acetic acid, both at the end of fermentation, and 150 days after the start of fermentation.

Microbiological analyses

The results of viable counts performed on the two prototypes of Kimchi made with sea fennel are presented in the table below.

Microbial group	Sampling time (days)	Started kimchi	Control kimchi
Mesophilic lactobacilli	t_0	$7.3 \pm 0.1^{b,A}$	$1.9 \pm 0.1^{d,B}$
	t_2	$7.5 \pm 0.1^{b,A}$	$3.5 \pm 0.3^{c,B}$
	t_5	$7.6 \pm 0.2^{b,A}$	$5.1 \pm 0.5^{b,B}$
	t_{12}	$8.3 \pm 0.0^{a,A}$	$8.2 \pm 0.1^{a,A}$
	t_{19}	$8.4 \pm 0.1^{a,A}$	$8.5 \pm 0.1^{a,A}$
	t_{26}	$8.4 \pm 0.1^{a,A}$	$8.5 \pm 0.0^{a,A}$
Mesophilic lactococci	t_0	$7.3 \pm 0.1^{b,A}$	$4.9 \pm 0.1^{b,B}$
	t_2	$7.4 \pm 0.0^{b,A}$	$5.9 \pm 0.3^{a,B}$
	t_5	$7.6 \pm 0.2^{b,A}$	$5.8 \pm 0.3^{a,B}$
	t_{12}	$8.3 \pm 0.0^{a,A}$	$6.6 \pm 0.3^{a,B}$
	t_{19}	$8.4 \pm 0.1^{a,A}$	$6.0 \pm 0.5^{a,B}$
	t_{26}	$8.3 \pm 0.1^{a,A}$	$6.1 \pm 0.4^{a,B}$
Yeasts	t_0	$< 1.0^{a,A}$	$< 1.0^{a,A}$

	t_2	< 1.0^{a,A}	< 1.0^{a,A}
	t_5	< 1.0^{a,A}	< 1.0^{a,A}
	t_{12}	< 1.0^{a,A}	< 1.0^{a,A}
	t_{19}	< 1.0^{a,A}	< 1.0^{a,A}
	t_{26}	< 1.0^{a,A}	< 1.0^{a,A}
Enterobacteriaceae	t_0	4.4 ± 0.5^{a,A}	4.3 ± 0.4^{a,A}
	t_2	5.6 ± 0.2^{a,A}	5.7 ± 1.0^{a,A}
	t_5	5.5 ± 0.8^{a,A}	5.1 ± 0.2^{a,A}
	t_{12}	4.4 ± 0.8^{a,B}	5.7 ± 0.2^{a,A}
	t_{19}	1.9 ± 0.7^{b,A}	3.1 ± 0.7^{b,A}
	t_{26}	< 1.0^{c,A}	< 1.0^{c,A}
Mesophilic aerobic bacteria	t_0	7.2 ± 0.0^{c,A}	5.4 ± 0.1^{b,B}
	t_2	7.5 ± 0.1^{bc,A}	6.2 ± 0.7^{b,B}
	t_5	7.6 ± 0.3^{b,A}	5.8 ± 0.3^{b,B}
	t_{12}	8.3 ± 0.0^{a,A}	8.3 ± 0.1^{a,A}
	t_{19}	8.4 ± 0.1^{a,A}	8.6 ± 0.1^{a,A}
	t_{26}	8.4 ± 0.0^{a,A}	8.4 ± 0.2^{a,A}
Pseudomonadaceae	t_0	5.2 ± 0.3^{a,A}	5.2 ± 0.1^{a,A}
	t_2	5.0 ± 0.3^{a,A}	5.7 ± 0.6^{a,A}
	t_5	4.9 ± 0.3^{a,A}	5.1 ± 0.6^{a,A}
	t_{12}	3.4 ± 0.3^{b,B}	5.2 ± 0.2^{a,A}
	t_{19}	2.5 ± 0.6^{bc,A}	2.8 ± 0.3^{b,A}
	t_{26}	2.1 ± 0.4^{c,A}	2.7 ± 0.6^{b,A}

Values are expressed as Log CFU g^{-1} ± standard deviation of three biological replicates. Within each row, for each microbial group at the same sampling time, overall means with different capital superscript letters are significantly different ($p < 0.05$). For each microbial group and sample, overall means with different small superscript letters in the same column are significantly different ($p < 0.05$).

The counts of mesophilic aerobic bacteria showed a slight difference during the first week 5.4 ± 0.1 and 7.2 ± 0.0 Log CFU g^{-1} for non-started and started Kimchi respectively, and the counting was stable until the end of the fermentation showing a same trend in both prototypes, with no significant differences at the end of fermentation where the microbial load reported was 8.4 Log CFU g^{-1} in both of them. .
Regarding mesophilic lactococci, a progressive increase in the load was reported during fermentation, where the highest counts were observed between day 5 and day 26 for both prototypes. Moreover, mesophilic lactococci counting was higher in started Kimchi than the non-started prototype at the end of fermentation with 8.3 ± 0.1 and 6.1 ± 0.4 Log CFU g^{-1}.
Regarding yeasts, counts reported were < 1.0 Log CFU g^{-1} in both prototypes.
Enterobacteriaceae counts decreased during fermentation period for both prototypes to reach <1.0 Log CFU g^{-1}at the end of fermentation.
As for Mesophilic aerobic bacteria, a similar trend is observed for both Kimchi prototypes described by an increase from day 5 to end of fermentation while the results showed that at the beginning of fermentation the counts in started Kimchi were higher than the non-started Kimchi.
Regarding Pseudomonadaceae, there were no significant differences between the two prototypes during the fermentation and the counts progressively decreased to reach 2.7 ± 0.6 and 2.1 ± 0.4 at the end of fermentation for started and non-started Kimchi respectively.
Finally, Coagulase-positive staphylococci and sulfite-reducing bacteria were under the detection limit (<1 Log CFU g^{-1}) in both prototypes. Coagulase-positive staphylococci and sulfite-reducing bacteria were not detected in both prototypes at the end of fermentation.

Identification of lactic acid bacteria

68 isolates were identified during the different sampling time. Among them, 54 were identified as lactic acid bacteria. The closest relatives, percentage identities, and accession numbers of the sequences obtained from the 54 lactic acid bacteria isolated from control Kimchi are reported in the table below.
In detail, the results showed that *Weissella koreensis* was the most abundantly detected species (22), followed by *Leuconostoc mesenteroides (17), Latilactobacillus graminis, (5), Leuconostoc citreum (4), Enterococcus hirae (3), Pediococcus pentosaceus (1),* and *Leuconostoc holzapfelii (1).*
The isolates were subjected to various characterization tests to identify the most effective strains for potential application in vegetable-based fermentations.

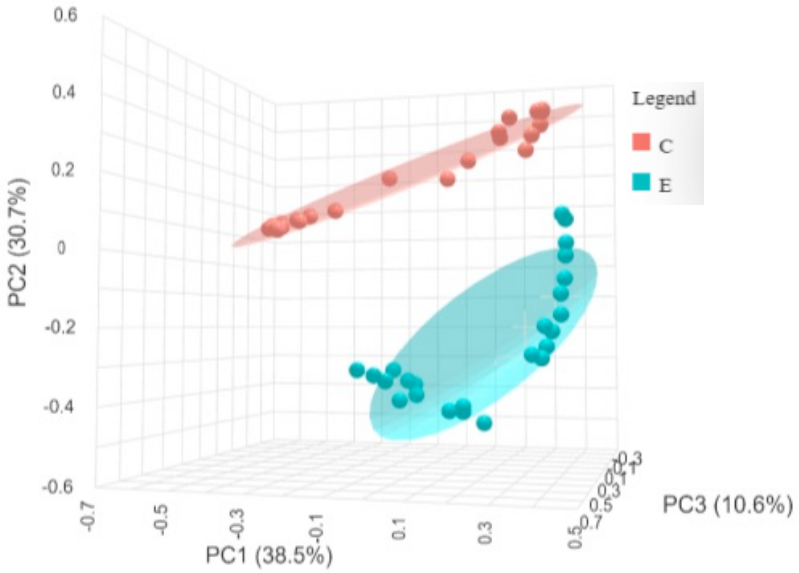
Identification of non-starter lactic acid bacteria (NSLAB) isolated from non-started Kimchi

Isolation source	Isolate code	Closest relative	% identity*	Accession number**
Kimchi	CK13	*Enterococcus hirae*	99.42%	NR_114783.2
Kimchi	CK14	*Enterococcus hirae*	99.89%	NR_114743.1
Kimchi	CK15	*Enterococcus hirae*	98.05%	NR_114783.2
Kimchi	CK18	*Pediococcus pentosaceus*	98.91%	NR_042058.1
Kimchi	CK28	*Weissella koreensis*	98.90%	NR_029041.1
Kimchi	CK29	*Weissella koreensis*	97.98%	NR_029041.1
Kimchi	CK30	*Weissella koreensis*	99.34%	NR_029041.1
Kimchi	CK32	*Weissella koreensis*	99,05%	NR_029041.1
Kimchi	CK31	*Weissella koreensis*	99.65%	NR_029041.1
Kimchi	CK33	*Weissella koreensis*	99.33%	NR_029041.1
Kimchi	CK35	*Weissella koreensis*	98.10%	NR_029041.1
Kimchi	CK36	*Weissella koreensis*	99.47%	NR_029041.1
Kimchi	CK46	*Leuconostoc mesenteroides*	98,57%	NR_074957
Kimchi	CK47	*Leuconostoc citreum*	98.28%	NR_041727.1
Kimchi	CK48	*Leuconostoc mesenteroides*	99.6%	NR_074957
Kimchi	CK49	*Weissella koreensis*	99.28%	NR_029041.1
Kimchi	CK51	*Weissella koreensis*	97.89%	NR_029041.1
Kimchi	CK52	*Leuconostoc citreum*	98.88%	NR_041727.1
Kimchi	CK53	Leuconostoc holzapfelii	98.63%	NR_042620

Kimchi	CK54	*Weissella koreensis*	99.61%	NR_029041.1
Kimchi	CK64	*Leuconostoc mesenteroides*	99.91%	NR_074957
Kimchi	CK65	*Leuconostoc mesenteroides*	99.42%	NR_074957
Kimchi	CK66	*Leuconostoc mesenteroides*	99.91%	NR_074957
Kimchi	CK67	*Leuconostoc mesenteroides*	97.45%	NR_074957
Kimchi	CK69	*Leuconostoc mesenteroides*	99.92%	NR_074957
Kimchi	CK70	*Leuconostoc mesenteroides*	97.36%	NR_074957
Kimchi	CK71	*Leuconostoc mesenteroides*	99,43%	NR_074957
Kimchi	CK72	*Leuconostoc mesenteroides*	98.74%	NR_157602
Kimchi	CK82	*Weissella koreensis*	99.63%	NR_029041.1
Kimchi	CK84	*Leuconostoc mesenteroides*	99.83%	NR_074957
Kimchi	CK85	*Weissella koreensis*	99,65%	NR_029041.1
Kimchi	CK86	*Leuconostoc mesenteroides*	97,76%	NR_113912.1
Kimchi	CK88	*Weissella koreensis*	99.34%	NR_029041.1
Kimchi	CK89	*Weissella koreensis*	99.08%	NR_029041.1
Kimchi	CK90	*Leuconostoc mesenteroides*	98.28%	NR_074957
Kimchi	CK93	*Leuconostoc citreum*	99,74%	NR_041727.1
Kimchi	CK94	*Leuconostoc mesenteroides*	97.65%	NR_074957
Kimchi	CK96	*Leuconostoc mesenteroides*	100.00%	NR_118557
Kimchi	CK100	*Leuconostoc mesenteroides*	99.82%	NR_074957
Kimchi	CK102	*Leuconostoc mesenteroides*	99.32%	NR_074957
Kimchi	CK103	*Leuconostoc mesenteroides*	99,91%	NR_113912.1
Kimchi	CK109	*Weissella koreensis*	97.66%	NR_029041.1
Kimchi	CK110	*Weissella koreensis*	98.68%	NR_029041.1
Kimchi	CK113	*Weissella koreensis*	99.55%	NR_029041.1
Kimchi	CK114	*Leuconostoc citreum*	99.66%	NR_041727.1
Kimchi	CK200	*Latilactobacillus graminis*	99.50%	NR_042438.1
Kimchi	CK201	*Latilactobacillus graminis*	99.92%	NR_042438.1
Kimchi	CK202	*Weissella koreensis*	99.65%	NR_029041.1
Kimchi	CK203	*Weissella koreensis*	98.47%	NR_029041.1
Kimchi	CK205	*Latilactobacillus graminis*	100.00%	NR_042438.1
Kimchi	CK204	*Latilactobacillus graminis*	98.12%	NR_042438.1
Kimchi	CK206	*Weissella koreensis*	99.73%	NR_029041.1
Kimchi	CK207	*Latilactobacillus graminis*	99.7%	NR_042438.1
Kimchi	CK208	*Weissella koreensis*	99.05%	NR_029041.1

Microbiota composition

A total of 136,483 bacterial reads were analyzed, with an average of approximately 2,904 reads per sample. For the fungal biota, a total of 2,332,367 reads were analyzed, with an average of 72,886 reads per sample. No statistically significant differences were observed in the diversity indices ($p > 0.05$), except for the Bray-Curtis dissimilarity index of bacterial ASVs, which differed significantly between the control (C) and experimental (E) kimchi groups ($p < 0.05$) as sh.

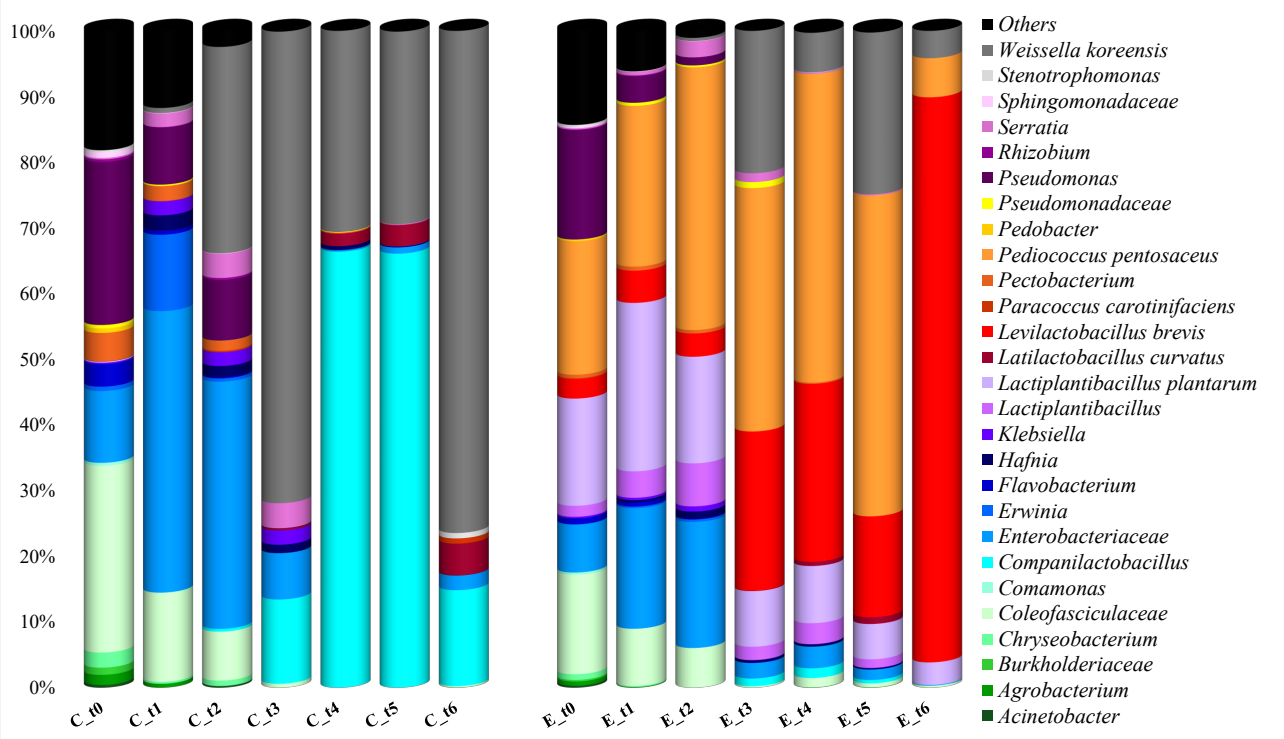


PCoA based on Bray-Curtis index of bacterial ASVs.

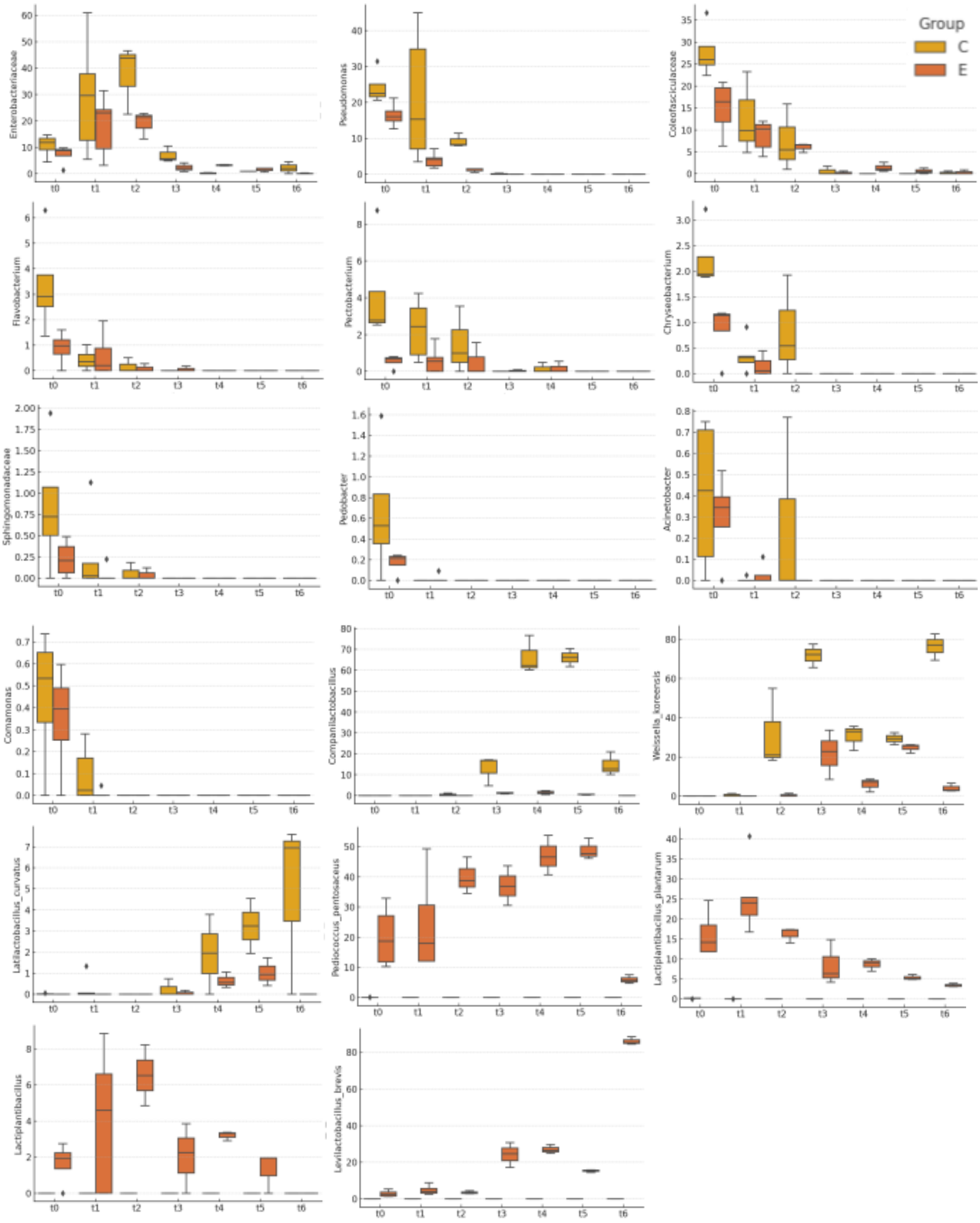
The bacterial biota composition of control and experimental kimchi is shown in Figure 2. Figure 3 illustrates box plots of taxa exhibiting statistically significant differences among samples ($p < 0.05$).

During the early fermentation stages (t0–t2), both control (C) and experimental (E) kimchi samples showed the occurrence of Enterobacteriaceae, *Pseudomonas* spp., Coleofasciculaceae, *Erwinia* spp., *Serratia* spp., and *Klebsiella* spp.. However, these taxa declined sharply after t2 and were consistently more abundant in the control group (overall mean of 30.37% *vs* 14.89% for *Enterobacteriaceae*; 14.07% *vs* 7.25% for *Pseudomonas* spp.; 16.25% *vs* 9.91% for Coleofasciculaceae; 4.24% *vs* 0.23% for Erwinia spp.; 1.99% *vs* 1.06% for *Serratia* spp.; 1.47% vs 0.45% for *Klebsiella* spp. in C and E, respectively). Several minor taxa, including *Acinetobacter* spp., *Agrobacterium* spp., *Chryseobacterium* spp., *Comamonas* spp., *Flavobacterium* spp., *Pectobacterium* spp., *Pedobacter* spp., and Sphingomonadaceae, were also more abundant in the control group during early fermentation, while being significantly lower or nearly absent in the experimental group.

As fermentation progressed, lactic acid bacteria (LAB) became dominant in both control and experimental kimchi. However, their succession and composition differed markedly. The experimental kimchi (E) exhibited an earlier and more stable LAB colonization. In detail, *Pediococcus pentosaceus*, *Lactiplantibacillus plantarum*, and *Levilactobacillus brevis* were predominant, with mean relative abundances of 31.90%, 11.99%, and 23.44%, respectively, from t_0 through t_6. Notably, *L. brevis* became the dominant species in the final stage of fermentation, reaching 86.03% of the relative abundance.



Bar plots illustrating the relative abundance of bacterial taxa at the finest taxonomic resolution across kimchi samples. Samples labeled "C" represent control kimchi, while "E" denotes experimental kimchi. Sampling times are as follows: t_0 – immediately after preparation; t_1 – 2 days; t_2 – 5 days; t_3 – 12 days; t_4 – 19 days; t_5 – 26 days; t_6 – 150 days



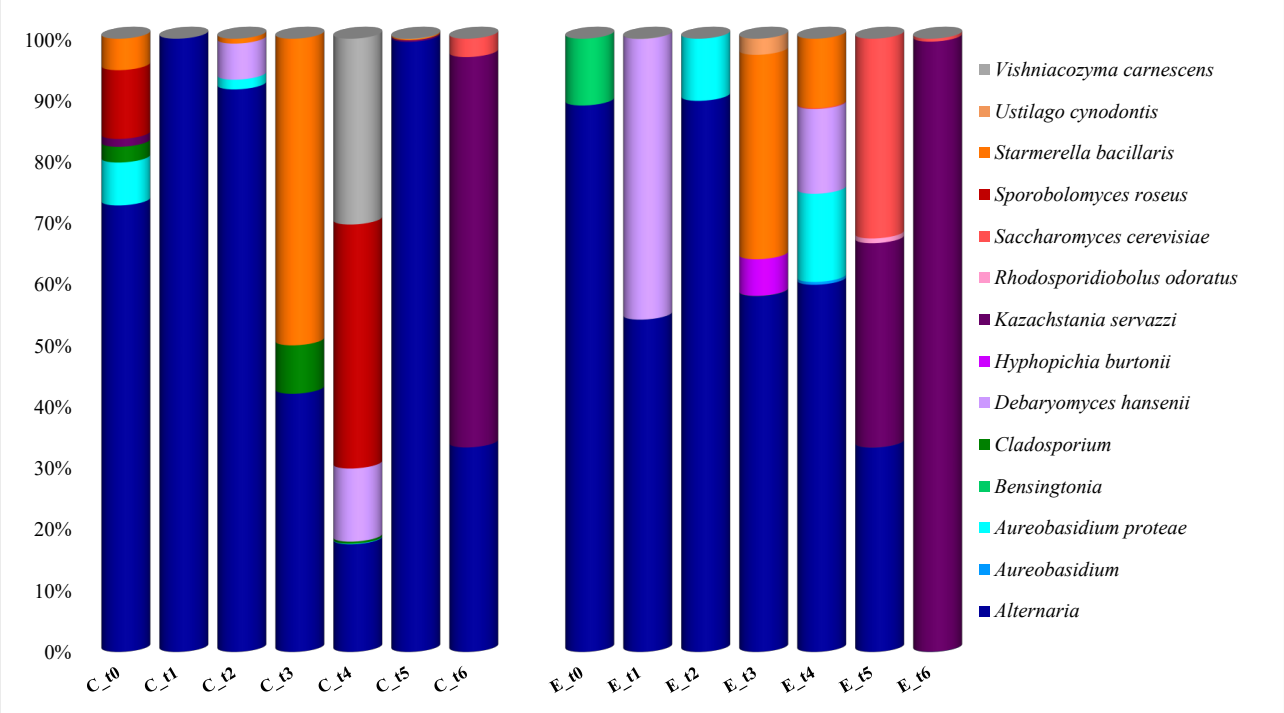
Box plots of bacterial taxa with statistically significant differences among samples ($p < 0.05$).

In contrast, the control group showed a delayed LAB succession. LAB dominance emerged at t_2, with *Weissella koreensis* reaching 31.43% of the relative abundance. This species persisted in both C and E groups through t_6 but remained significantly more abundant in the control samples (47.90% in C *vs* 11.39% in E). *Companilactobacillus* spp. emerged at t3 in the control group (12.83%), peaked at t4–t5 (66%), and settled at 14.60% by t_6. *Latilactobacillus curvatus* also increased slightly from t_3 to t_6, reaching 4.84% by the end of fermentation. These taxa appeared only sporadically in the experimental samples during t_3–t_5, each accounting for less than 2% of the relative abundance.

The composition of the fungal microbiota in control and experimental kimchi samples is presented in Figure 4.

Alternaria spp. was the most prevalent taxon across all samples, with overall mean relative abundances of 65.30% and 54.91% in control and experimental kimchi, respectively.

While the overall fungal profiles exhibited considerable variability depending on both fermentation type (C *vs* E) and sampling time, statistical analysis revealed significant differences ($p < 0.05$) for only two taxa. In detail, *Bensingtonia* spp. was detected primarily during the early stages of fermentation, with the highest relative abundance observed in experimental samples at t_0 (E_t_0). In contrast, *Kazachstania servazzii* became prominent in the later stages, particularly in the inoculated (E) kimchi. Several taxa, including *Aureobasidium proteae*, *Debaryomyces hansenii*, *Saccharomyces cerevisiae*, and *Starmerella bacillaris*, appeared sporadically in both control and experimental samples without a consistent pattern. The control group also exhibited variable occurrences of *Cladosporium* spp., *Sporobolomyces roseus*, and *Vishniacozyma carnescens*, whereas the experimental group showed only rare detection of *Aureobasidium* spp., *Hyphopichia burtonii*, *Rhodosporidiobolus odoratus*, and *Ustilago cynodontis*.



Bar plots illustrating the relative abundance of fungal taxa at the finest taxonomic resolution across kimchi samples. Samples labeled "C" represent control kimchi, while "E" denotes experimental kimchi. Sampling times are as follows: t_0 – immediately after preparation; t_1 – 2 days; t_2 – 5 days; t_3 – 12 days; t_4 – 19 days; t_5 – 26 days; t_6 – 150 days.

Volatile components analysis

Results of semi-quantitative analysis of Volatile Organic compounds (VOCs) detected in the static headspace of the two prototypes of Kimchi made with sea fennel are reported in Table 6. The analysis allowed the identification of 38 compounds including (4) Aldehydes: Acetaldehyde, Pentanal, Geranial, Neral; (3) Esters: Ethyl Acetate, 1-Butanol-3-methyl acetate, 1-Methoxy-2-propyl acetate; (6) Alcohols: Ethanol, 1-Penten-3-ol, 3-Methyl-1-butanol, (Z)-3-Hexen-1-ol, 1-Pentanol, Phenylethyl alcohol; (3) Carboxylic Acids: Acetic acid, Hexanoic acid, Octanoic acid; (15) Terpenes: Camphene, α-Pinene, β-Myrcene, Sabinene, γ-Terpinene, D-Limonene, β-Phellandrene, p-Cymene, α-Terpineol, Terpinene-4-ol, Borneol, 1,8-Cineol, Camphor, Thymol methyl ether, Dill apiole; (2) Sulfur compounds: Dimethyl trisulfide, Dimethyl disulfide; (2) Nitriles: 5-Cyano-1-pentene, Benzene propanenitrile; (1) Phenols: Carvacrol; (1) Heterocyclic compounds: unknown Thiazole; (1) Isothocyonates: Phenethyl Isothiocyanate.

Results showed a generally similar trend between control and started kimchi made with sea fennel, with only minor variation in specific compounds. Acetaldehyde, detected at the beginning of fermentation (t_0), was absent at the end of fermentation and after 150 days. Similarly, sabinene showed high concentration at the early stage of fermentation but decreased significantly, reaching negligible level in both prototypes.

During fermentation, the level of dimethyl disulfide, acetic acid, dill apiole, α-terpineol, borneol, benzene propanenitrile, p-cymene, 4-ethyl-5-methylthiazole, terpinene-4-ol and phenethyl isothiocyanate increased significantly from t_0 to t_{26}. Among these, p-cymene and terpinene-4-ol were the major compounds. Terpinene-4-ol reached the highest chromatographic peak area of 239.30 ± 41.02 and 249.58 ± 28.29 ×10^5 at t_{26}, for control and started Kimchi, respectively. However, dillapiole was more abundant in control Kimchi, whereas acetic acid was higher in the started Kimchi.

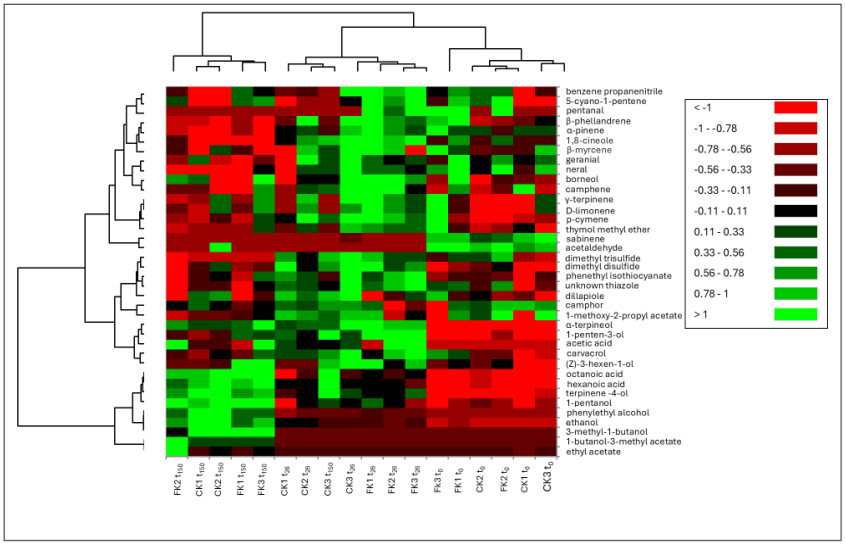
Additionally, compounds such as ethanol, 1-pentanol, carboxylic acids (hexanoic acid, octanoic acid), 3-methyl-1-butanol, ethyl acetate and terpinene-4-ol increased significantly after 150 days since the start of fermentation. Ethanol, 3-methyl-1-butanol, and terpinene-4-ol concentrations reached chromatographic peak areas of 175.14, 275.84, 451.32 ×10^5, respectively, for control Kimchi, and 124.80, 200.60, 333.30 ×10^5 for started kimchi, respectively.

Terpenes such as γ-terpinene and β-myrcene, camphor, thymol methyl ether and D-limonene, along with other compounds, like 1-methoxy-2-propyl acetate, remained stable throughout fermentation process. Phenolic compounds like Carvacrol showed minimal variation over monitoring time in both treatments.

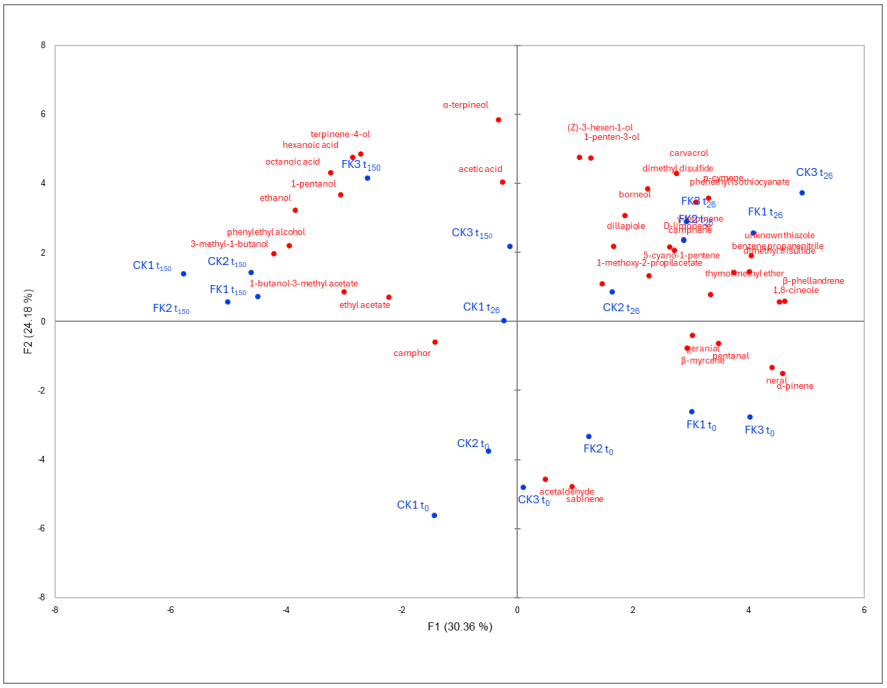
The heatmap illustrates the concentration of VOCs across replicates and fermentation time in both prototypes. Very low concentrations correspond to red, while very high concentrations correspond to light green colour. The analysis revealed a separation of samples based on sampling time. In fact, samples from t_0 were clustered together, as did those from t_{26} and t_{150} except CK5 (t_{150}). At the beginning of fermentation (t_0), sabinene and acetaldehyde, were more abundant regardless of the use of starter. At t_{26}, Kimchi prototypes produced significantly higher concentration of terpenes such as β-phellandrene, α-pinene, D-Limonene and p-Cymene along with acetic acid in most of the replicates, indicating its accumulation over time. At t_{150}, the VOC profile in started and control Kimchi was enriched with terpinene-4-ol and alcohols such as (Z)-3-hexen-1-ol, 1-pentanol, 3-methyl-1-butanol and ethanol, as well as carboxylic acids like octanoic and hexanoic acid in samples FK1, FK2, FK3, CK1 and CK2 t_{150}. Other noteworthy compounds were also observed at this stage of the monitoring period, such as 3-methyl-1-butanol and ethyl acetate.

To confirm the correlation between the volatile compounds, fermentation time and treatment, a PCA analysis was conducted as shown in the figure below. The figure displays the biplot of the score plot and the loading plot from the PCA performed on the quantitative data of VOCs. PC1 and PC2 explained 30 and 24% of the total variance accounting for a cumulative 54.54% of the variability in the dataset. PC1 neatly separated samples analyzed at t_{150}, CK1(t_{26}), CK2 and CK1 (t_0), all characterized by negative scores, from all the other samples collected at t_{26} and t_0, characterized by positive scores. PC2, distinguished samples from early fermentation (t_0) exhibiting negative scores from those sampled at t_{26} and t_{150}. Samples clustered according to their fermentation stage, rather than the application of starter culture, forming distinct clusters in different quadrants and indicating a significant difference between them. Regarding metabolite distribution, Acetaldehyde and monoterpenes were prominent at t_0, while compounds such as phenols, alcohols, esters and carboxylic acids were strongly associated with samples from later stages of fermentation (t_{26} and t_{150}).

In more detail, at the beginning of fermentation (t_0), the samples CK1, CK2, CK3, FK1, FK2, and FK3 were associated with aldehydes and monoterpenes. By the end of fermentation, the replicates CK2, CK3, FK1, FK2 and FK3 t_{26} were linked to terpenes, alcohol, sulfur-containing compounds and phenols. Conversely, the replicate $CK2t_{26}$ showed a distinct VOC profile, compared to other control Kimchi samples at t_{26}. However, the VOC profile shifted at t_{150}, characterized by abundance of carboxylic acids and alcohols including acetic acid, ethanol, octanoic and hexanoic acid.



Plot of Heat Map analysis performed on control and started Kimchi made with sea fennel (38 VOCs in 18 Kimchi samples).
Note. Labels of Kimchi samples represent replicates of started Kimchi (FK) and control Kimchi (CK), followed by replicate numbers (1; 2; 3) and sampling time (t_0; t_{26}; t_{150}).

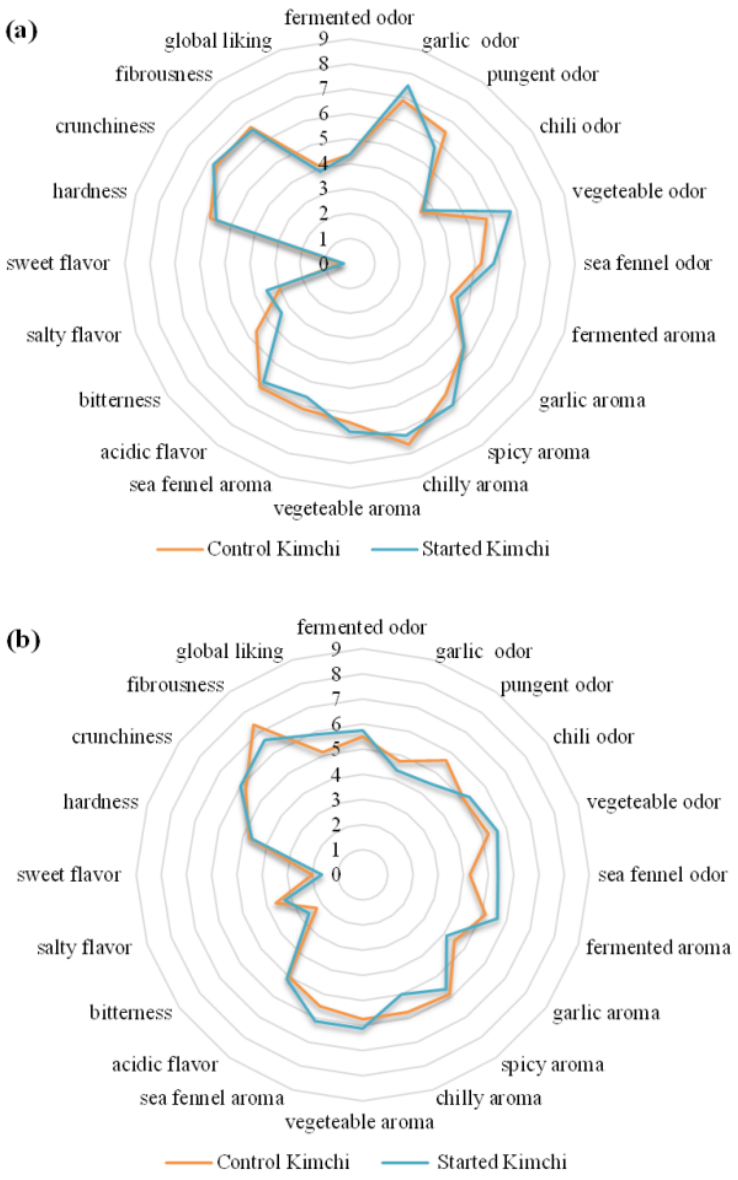


Principal Component Analysis bi-plot of the first two PCs obtained on VOCs whole dataset.

Note. Observations, in blue labels, refer to Kimchi samples: labels indicate the type of Kimchi (FK for started Kimchi, CK for control Kimchi), followed by replicate numbers (1; 2; 3) and sampling time (t_0; t_{26}; t_{150}). Variables, in red labels, refer to individual VOCs.

Sensory analyses

Overall, most sensory attributes were perceived similarly between the two prototypes, regardless of the use of a starter culture. Spiciness, chili aroma, and garlic odor were more strongly perceived in all samples at day 26 (t26) (Figure 3, panel a) compared to day 150 (t150) (Figure 3, panel b). No significant differences in crunchiness were observed between the two samples at either point, although fibrousness slightly increased in both after 150 days of storage.The sea fennel flavor was perceived as slightly more intense in the starter-inoculated kimchi than in the control at t150. Both samples received relatively low scores for sweetness and saltiness at both time points. Regarding overall acceptability, the starter-inoculated kimchi was rated more favorably than the naturally fermented counterpart at t150, with the highest score being 5.88 ± 0.64.



Results of sensory analysis performed on started and control kimchi. (a) Sensory analyses performed after 26 days of fermentation; (b) sensory analyses performed after 150 days since the start of fermentation. Each sample was evaluated by a trained panel consisting of 8 non-smoker tasters aged between 25 and 48 for the presence and intensity of (i) six olfactory descriptors, being fermented, garlic, pungent, chilly, vegetable and sea fennel; (ii) six aroma descriptors, being fermented, garlic, spicy, chilly, vegetable and sea fennel; (iii) four flavor descriptors, being acidity, bitterness, salty, and sweet; (iv) three textural descriptors, being hardness, fibrousness, and crunchiness; (v) global acceptance. Each descriptor was evaluated by attributing a score comprised between 1 and 9, with 1 expressing the lowest and 9 the highest intensity. Results are reported as mean values ± standard deviation.

References
Hynes WL, Ferretti JJ, Gilmore MS, Segarra RA., 1992. PCR amplification of streptococcal DNA using crude cell lysates. FEMS Microbiol Lett. 94(1–2):139–142,http://dx.doi.org/10.1016/0378-1097(92)90597-h.
Maoloni A, Milanović V, Osimani A, Cardinali F, Garofalo C, Belleggia L, Foligni R, Mannozzi C, Mozzon M, Cirlini M, et al., 2021. Exploitation of sea fennel (Crithmum maritimum L.) for manufacturing of novel high-value fermented preserves. Food Bioprod Process. 127:174–197,
http://dx.doi.org/10.1016/0378-1097(92)90597-h.
Osimani A, Garofalo C, Aquilanti L, Milanović V, Clementi F., 2015. Unpasteurised commercial boza as a source of microbial diversity. Int J Food Microbiol. 194:62–70,
http://dx.doi.org/10.1016/0378-1097(92)90597-h.
Cardinali, F., Botta, C., Harasym, J., Reale, A., Ferrocino, I., Boscaino, F., Orkusz, A., Milanović, V., Garofalo, C., Rampanti, G., Aquilanti, L., & Osimani, A. 2024. Tasting of traditional Polish fermented cucumbers: Microbiology, morpho-textural features, and volatilome. Food Research International, 177, 113851. https://doi.org/10.1016/j.foodres.2023.113851
Klindworth A, Pruesse E, Schweer T, Peplies J, Quast C, Horn M, GlöcknerFO. 2013. Evaluation of general 16S ribosomal RNA gene PCR primers forclassical and next-generation sequencing-based diversity studies. Nu-cleic Acids Res 41:e1. https://doi.org/10.1093/nar/gks808.34.
Mota-Gutierrez, J., Ferrocino, I., Rantsiou, K., & Cocolin, L. 2019. Metataxonomic comparison between internal transcribed spacer and 26S ribosomal large subunit (LSU) rDNA gene. International Journal of Food Microbiology, 290, 132–140.
Chong, J., Liu, P., Zhou, G., & Xia, J. 2020. Using MicrobiomeAnalyst for comprehensive statistical, functional, and meta-analysis of microbiome data. Nature protocols, 15(3), 799–821. https://doi.org/10.1038/s41596-019-0264-1
Fu, N., Woo, M. W., Selomulya, C., & Chen, X. D. (2013). Inactivation of Lactococcus lactis ssp. cremoris cells in a droplet during convective drying. Biochemical Engineering Journal, 79, 46–56.
https://doi.org/10.1016/j.bej.2013.06.015
Peryam, D. R., & Pilgrim, F. J. 1957. Hedonic scale method of measuring food preferences. Food Technology, 11(Suppl), 9–14.

1.3 Fermented sea fennel sprouts in vinegar

Material and Methods

Starter culture

The same starter strains used for the fermentation of kimchi have been used for the manufacture of laboratory-scale prototypes of fermented pickles.

Preparation of fermented sea fennel sprouts in vinegar

Laboratory scale prototypes of fermented sea fennel were produced by collecting a 5 kg sea fennel sprouts from the local farm (Rinci S.r.l, Castefidardo, Ancona, Italy) which cultivates sea fennel crops for food use industry. They were washed and rinsed with tap water, blanched at 95°C for 30s, and drained for 10 min by air-drying. Then 3 replicates were prepared, and each one consists of one plastic container containing a mix of 1795 g of blanched sea fennel with 5385 mL brine composed of autoclaved 7 % NaCl solution and 1 % fructose sterilized by filtration. All the replicates were inoculated by the 4 strains previously mentioned to reach 7 Log CFU mL^{-1} in brine. Two batches have been performed in the same way separately during November 2022 and July 2023.

Laboratory scale prototype of fermented sea fennel preserves produced by mixing blanched sea fennel sprouts and sterile brine.

Physical-chemical analyses (pH and TTA)

Aliquots (1 mL) of brine of each replicate were aseptically collected immediately after inoculation and during the fermentation until the end of the monitoring period corresponding to day 0, 1, 3, 6, 8, 10, 13, 15, 17 and 20. The pH measurement was accomplished with a pH meter model 300 (Hanna Instruments, Padova, Italy). The results were expressed as the mean of the replicates ± standard deviation.

Concerning TTA, the analyses were performed by aliquoting and blending 10 g of sea fennel sprouts for each replicate with 90mL of distilled water. The final suspension was titrated with 0.1 NaOH and the results expressed as % of lactic acid. TTA analyses were reported as mean of the 3 replicates ± standard deviation.

Microbial counting

Microbiological analyses were performed in brine samples. In more detail, counting of: (i) mesophilic aerobic bacteria, (ii) presumptive mesophilic lactobacilli, (iii) yeasts, and (iv) Enterobacteriaceae were performed as described previously for Kimchi in 1.2.1.4.

Tenfold serial dilutions were prepared from the brine of each inoculated sea fennel preserves replicate. The results of viable counting were expressed as the mean Log colony forming units (CFU) mL^{-1} of sea fennel preserves of three replicates ± standard deviation.

Statistical analysis

To assess statistical differences within Kimchi samples, the Tukey-Kramer's Honest Significant Difference (HSD) test (level of significance 0.05) was used by one-way analysis of variance (ANOVA). Tests were performed through JMP v11.0.0 software (SAS Institute Inc., Cary, NC).

Results

Physical-chemical characterization

The results of physical-chemical characterization of the analyzed prototype of fermented sea fennel preserve in batch 1 and 2 are reported in tables below.

Results of the pH determination of prototypes of fermented sea fennel.

Sampling time (t, days)	Prototypes	
	Batch 1	Batch 2
	Fermented Sea Fennel in brine	Fermented sea fennel in brine
t_0	6.05 ± 0.02^{a}	6.28 ± 0.05^{a}
t1	5.86 ± 0.06^{a}	5.32 ± 0.07^{b}
t_3	5.43 ± 0.10^{b}	5.08 ± 0.03^{c}
t_6	4.78 ± 0.19^{c}	4.91 ± 0.05^{c}
t_8	4.25 ± 0.20^{d}	4.86 ± 0.04^{cd}
t_{10}	3.83 ± 0.16^{e}	4.66 ± 0.09^{d}
t_{13}	3.80 ± 0.05^{e}	4.25 ± 0.08^{e}
t_{15}	3.62 ± 0.08^{e}	4.17 ± 0.09e
t_{17}	3.58 ± 0.12^{e}	4.07 ± 0.09^{e}
t_{20}	3.59 ± 0.06^{e}	4.04 ± 0.14^{e}

The results were expressed as the means of two independent measurements for each of the three replicates ± standard deviation. Within each row, for each batch overall means with different lowercase superscript letters are significantly different ($p < 0.05$).

Results of the titratable acidity determination of prototypes of fermented sea fennel.

Sampling time (t, days)	Prototypes	
	Batch 1	Batch 2
	Fermented sea fennel	Fermented sea fennel
t_0	0.12 ± 0.04^{a}	0.03 ± 0.00^{b}
t_{26}	0.18 ± 0.02^{a}	0.32 ± 0.03^{a}

The results are expressed as means % lactic acid of three replicates ± standard deviations. Within each row, for each batch overall means with different lowercase superscript letters are significantly different ($p < 0.05$).

The pH value decreased during the monitoring period in batch 1 and 2 where it was significantly lower at the end of fermentation which passed from 6.05 ± 0.02 to 3.59 ± 0.06 and from 6.28±0.05 to 4.04±0.14 respectively in batch 1 and 2, contrarily to TTA that increased from 0.12 ± 0.04 to 0.18 ± 0.02 and from 0.03±0.00 to 0.32±0.03 % lactic acid equivalent.

Microbial Viable Counts

Microbiological analyses were performed in brine samples of fermented sea fennel in batches 1 and 2 and theresults are reported below.

Microbial counting of fermented sea fennel preserves brine during fermentation process for Batch 1 and 2.

Microbial group	Sampling time (t, days)	Prototypes	
		Batch 1	Batch 2
Mesophilic lactobacilli (Log CFU mL^{-1})		Fermented sea fennel preserves	
	t_0	7.3 ± 0.0^{a}	7.2 ± 0.1^{a}
	t_1	6.4 ± 0.1^{b}	6.8 ± 0.0^{ab}
	t_3	6.4 ± 0.1^{b}	6.3 ± 0.0^{c}
	t_6	6.2 ± 0.2^{b}	6.5 ± 0.4^{bc}
	t_{13}	7.3 ± 0.1^{a}	6.7 ± 0.2^{abc}
	t_{20}	6.4 ± 0.2^{a}	6.9 ± 0.2^{ab}
Yeasts (Log CFU mL^{-1})			
	t_0	< 1.0^{c}	0.0 ± 0.2^{b}
	t_1	< 1.0^{c}	2.7± 0.1^{b}
	t_3	1.7 ± 0.4^{c}	4.6 ± 0.4^{a}
	t_6	4.1 ± 0.7^{b}	5.6 ± 0.1^{a}
	t_{13}	6.7 ± 0.2^{ab}	5.2 ± 1.2^{a}
	t_{20}	6.4 ± 0.1^{a}	5.3 ± 0.6^{a}
Enterobacteriaceae (Log CFU mL^{-1})			
	t_0	< 1.0b	0.4 ± 0.8^{c}
	t_1	< 1.0^{b}	0.3 ± 0.6^{c}
	t_3	4.5 ± 1.5^{a}	6.7 ± 0.5^{a}
	t_6	5.4 ± 1.2^{a}	6.5 ± 0.4^{a}
	t_{13}	< 1.0^{b}	3.6 ± 0.7^{b}
	t_{20}	< 1.0^{b}	2.4 ± 0.5^{b}
Mesophilic aerobic bacteria (Log CFU mL^{-1})			
	t_0	7.3 ± 0.0^{a}	7.1 ± 0.0^{a}
	t_1	6.3 ± 0.1^{bc}	6.7 ± 0.0^{ab}
	t_3	5.8 ± 0.3^{c}	6.9 ± 0.2^{ab}
	t_6	6.5 ± 0.4^{b}	7.1 ± 0.3^{a}
	t_{13}	6.3 ± 0.2^{bc}	6.9 ± 0.2^{ab}
	t_{20}	5.9 ± 0.3^{bc}	6.6 ± 0.1^{b}

The results are expressed as means of three replicates ± standard deviation. Within each row, for each microbial group in each batch, overall means with different lowercase superscript letters are significantly different ($p < 0.05$).

During the monitoring period of fermentation of fermented sea fennel preserves, the mesophilic lactobacilli slightly decreased at the end of fermentation for Batch 1 and 2 which passed from 7.3 ± 0.0 to 5.9 ± 0.3 Log CFU mL^{-1} and from 7.2 ± 0.1 to 6.9 ± 0.2 Log CFU mL^{-1} similarly to Mesophilic aerobic bacteria which had similar trend between the beginning and day the end of fermentation time.

Regarding Enterobacteriaceae, they disappeared at the end of fermentation in Batch 1 although they increased between day 3 and day 6 then decreased from day 6 to day 20 in batch 2 with reported values of 6.5 ± 0.4 to 2.4 and 5.3 ± 0.5 Log CFU mL^{-1}. The enumeration of the yeasts showed an increase during the monitoring period that reached 6.4 ± 0.1 and 5.3 ± 0.6 Log CFU mL^{-1} in batch 1 and 2 respectively.

1.3.1 PICKLES

The fermented sea fennel in brine has been processed into 24 pickles samples composed each of 300g vinegar and mineral water and 200g of fermented sea fennel leaves and sprouts with different acidity percentages. Two types of vinegar are used: wine and apple vinegar with 12 samples for each type including three replicates for each acidity level applied. The different acidity percentages are as follows: 0.05%, 0.2%, 0.5% and 0.7%.

The 24 samples have been subjected to the pasteurization mild thermal treatment equivalent to 74°C for 3min to kill and eliminate vegetative pathogenic organisms and to extend the shelf life of food product for a limited period.

The pickles are analyzed for a period of 6 months starting from May until November to assess their shelf-life and determine the optimal acidity level for preservation and organoleptic quality They are sampled during the monitoring period for pH, TTA, microbial enumeration, color and volatile compounds. The two batches were produced with two types of vinegar.

Pickles sample containing fermented sea fennel, water and vinegar.

Material and Methods

Physical-chemical analyses (pH and TTA)

Aliquots (1 mL) of brine of each replicate from each sample were aseptically collected immediately after pasteurization and during the monitoring period at the following months: 0, 1, 2, 3, 4, 5, and 6. The pH measurement was accomplished with a pH meter model 300 (Hanna Instruments, Padova, Italy). The results were expressed as mean of the three biological replicates for each technical ± standard deviation.

Concerning TTA, the analyses were performed by aliquoting 10 ml of pickle brine for each replicate with 90mL of distilled water. The final product was titrated 0.1 NaOH and the results expressed as % of lactic acid equivalent. TTA analyses were reported as mean of the 3 replicates ± standard deviation.

Microbial Viable Counts

Microbiological analyses were performed in brine pickle samples. In more details, counting of: (i) mesophilic aerobic bacteria, (ii) presumptive mesophilic lactobacilli, (iii) yeasts, and (iv) Enterobacteriaceae were performed as described previously for Kimchi.

Tenfold serial dilutions were prepared from the brine of each pickle replicate. The results of viable counting were expressed as the mean Log colony forming units (CFU) mL^{-1} of three replicates ± standard deviation.

Color assessment

The colorimetric test of pickles was performed on sea fennel leaves in each replicate, bounding together at least three leaves with the same size to create a homogenous sample. Color parameters in the CIELab color space, lightness (L), redeness-greeness (a*: + red; − green), and yellowness-blueness (b*: + yellow; − blue) were measured using a Chroma Meter CR-200 (Minolta Japan). In addition, the hue angle h* (h°) was calculated using the formula h° = 180 + arctg (b*/a*) (Mclellan et al., 1995), and the chroma (C) was calculated using the formula $C = [(a^{*2} + b^{*2})]^{1/2}$. The results were expressed as the mean of three replicates per sample ± standard deviation.

Salt determination

Sea fennel leaves (5 g) were homogenized in 20 g of distilled water using an Ultra-Turrax machine at an estimated speed of 10,000 RPM for 2 minutes, ensuring thorough mixing for subsequent analysis. Then, Salt measurements were conducted at two time points: the initial month (Month 0) and six months later (Month 6). However, samples prepared with 0.05% acidity were excluded from the comparison, as they were contaminated and could not be reliably evaluated against the initial month.

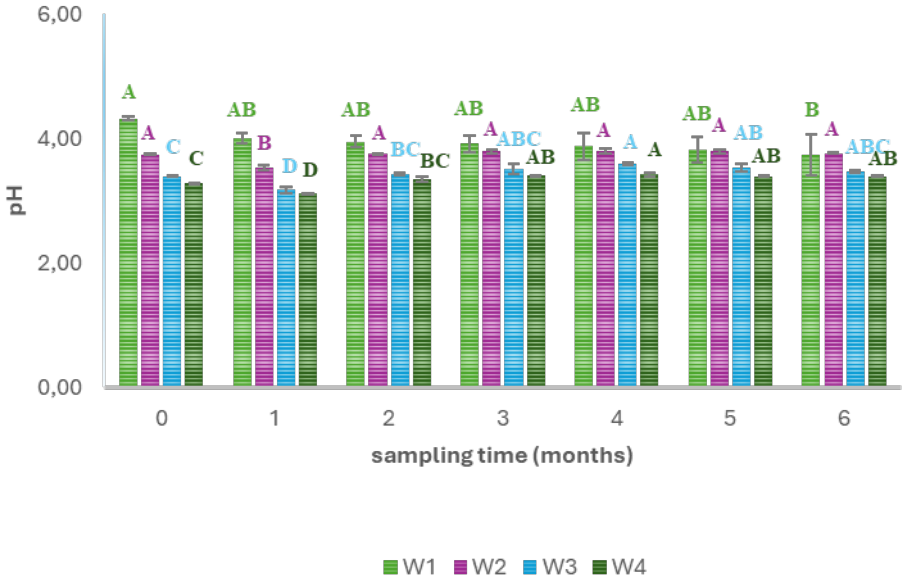
Sensory analysis

Sensory analysis was conducted to evaluate the organoleptic properties of the sea fennel pickles samples over time. attributes such as herbal, woody, acidic, and sea fennel odors; herbal, woody, acidic, and sea fennel aromas; acidic flavor, bitterness, salty flavor, and sweet flavor; as well as textural attributes like hardness, fibrosity, and crunchiness, were analyzed alongside an overall assessment of global liking. Samples with 0.05% acidity were excluded from the analysis due to contamination, which prevented reliable comparisons.

Results

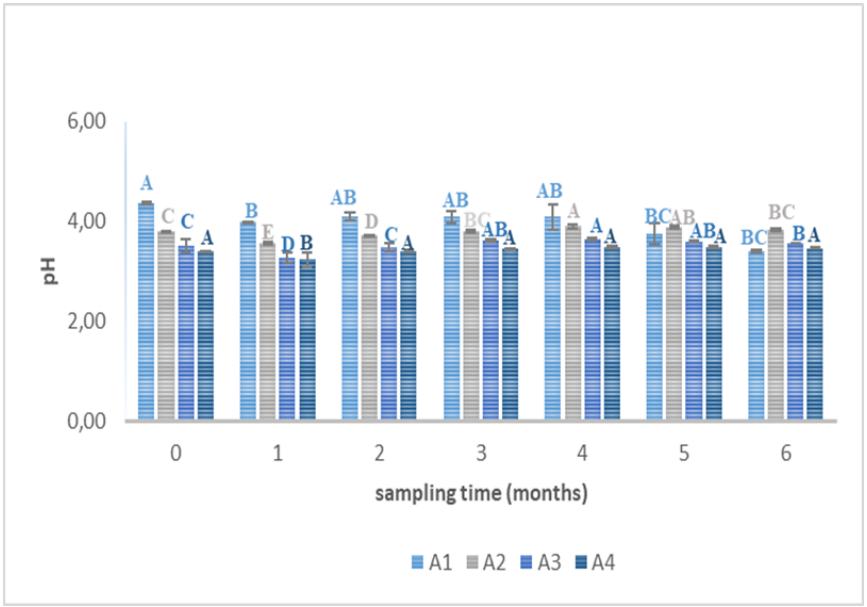
Physical-chemical characterization

The results of pH determination of the analyzed prototype of pickles made of wine and apple vinegar in batch 1 are demonstrated in the figures below.



Results of the pH determination of pickles prototypes made of sea fennel and wine.

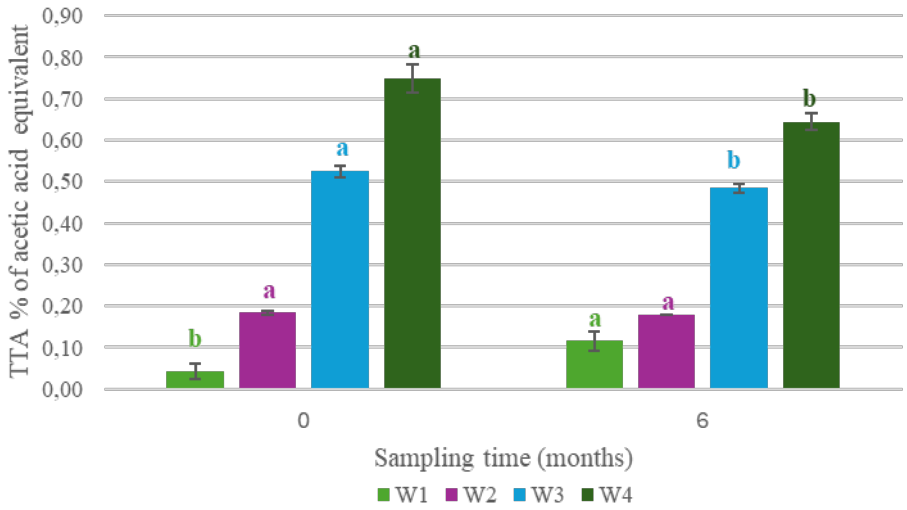
W1: samples with 0.05% acidity (total acetic acid in the final product)
W2: samples with 0.2% acidity
W3: samples with 0.5% acidity
W4: samples with 0.7% acidity
Results are expressed as mean of three biological replicates for each sample ± standard deviation.



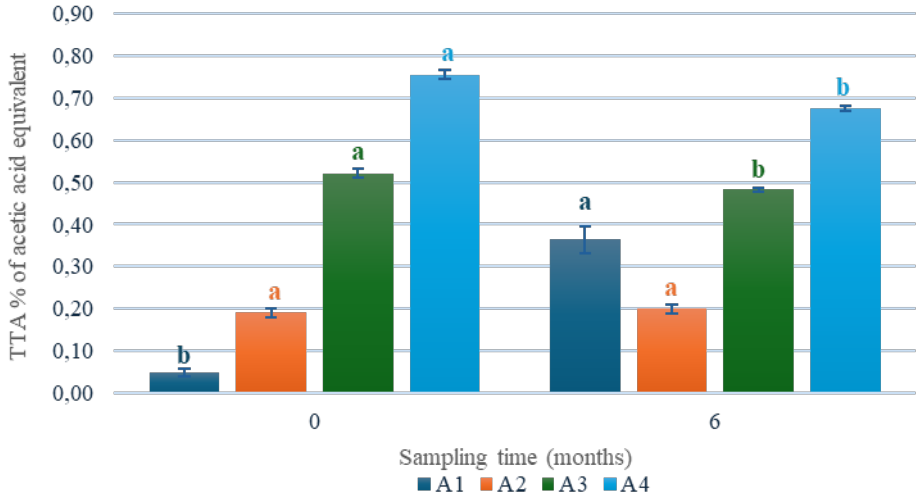
Results of the pH determination of pickles prototypes made of sea fennel and apple vinegar.

A1: samples with 0.05% acidity (total acetic acid in the final product)
A2: samples with 0.2% acidity
A3: samples with 0.5% acidity
A4: samples with 0.7% acidity
Results are expressed as mean of three biological replicates for each sample ± standard deviation.

The pH values remained relatively stable for both prototypes, whether using wine or apple vinegar during the monitoring period except the samples with the lowest acidity (0.05%) where the pH values decreased from t0 to t6, from 4.32 ± 0.02 to 3.73 ± 0.34 and from 4.36 ± 0.02 to 3.41 ± 0.02 for W1 and A1, respectively. As for the remaining samples made with the wine vinegar, the pH at the end of monitoring period (t6) ranged from 3.39 ± 0.02 to 3.77 ± 0.01, varying according to the acidity level applied. However, in the samples made with apple vinegar, pH ranged from 3.46 ± 0.02 to 3.83 ± 0.02.



Results of TTA in sea fennel pickles made with wine vinegar.



Results of TTA measurements in sea fennel pickles made with apple vinegar.
The results were expressed as the means of three biological replicates ± standard deviation.

Based on the reported results in table 2, the total titratable acidity (TTA) increased with higher acidity levels of the vinegars used. Moreover, minimal variation was observed among the different prototypes except for W1 and A1 where TTA values progressively increased. At t0, TTA ranged from 0.06 ± 0.02 to 1.12 ± 0.03 % of acetic acid equivalent in wine vinegar pickles and from 0.07 ± 0.01 to 1.13 ± 0.01 % of acetic acid equivalent in apple vinegar pickles, correlating with the acidity levels. However, at t6, TTA values ranged from 0.17 ± 0.02 to 0.97 ± 0.02 for pickles made with wine vinegar and from 0.54 ± 0.03 to 1.01 ± 0.01 % of acetic acid equivalent, for apple vinegar pickles.

In conclusion, except for the samples with 0.05% acidity, the pH of the samples made with either apple wine vinegar exhibited an opposite trend to the acidity level applied. Additionally, the Total Titratable Acidity (TTA) values increased proportionally with the acidity of the vinegar used.

Microbial enumeration

Overall, mesophilic lactobacilli were generally not detected in batch 1 and 2 in most of the samples during the six months monitoring period. Mesophilic aerobic bacteria showed limited growth in the samples made with acidity higher than 0,05%, while the growth reached 4.61 to 4.69 Log CFU/mL in both batches in W1 and A1. Similarly, Yeasts showed a gradual growth from month 3 in samples A1 and W1 regardless of number of batches. However, Enterobacteriaceae were absent throughout the shelf-life study.
In summary, both batches demonstrated microbial stability, with specific aerobic and yeast growth linked to acidity levels, and no presence of harmful bacteria was observed.
Coagulase-positive staphylococci (*Staphylococcus aureus* and other species), reducing anaerobic bacteria, Spores of sulfite-reducing anaerobic bacteria, and *Listeria monocytogenes* were not detected in both batches at time 0 and 6.

Microbial counting of pickled sea fennel in vinegar Batch 1.

Microbiological group	Sampling time (months)	Prototypes							
Mesophilic lactobacilli		W1	W2	W3	W4	A1	A2	A3	A4

(Log CFU mL^{-1})									
	t0	< 1.0	< 1.0	< 1.0	< 1.0	< 1.0	< 1.0	< 1.0	< 1.0
	t1	< 1.0	< 1.0	< 1.0	< 1.0	< 1.0	< 1.0	< 1.0	< 1.0
	t2	< 1.0	< 1.0	< 1.0	< 1.0	< 1.0	< 1.0	< 1.0	< 1.0
	t3	< 1.0	< 1.0	< 1.0	< 1.0	< 1.0	< 1.0	< 1.0	< 1.0
	t4	< 1.0	< 1.0	< 1.0	< 1.0	< 1.0	< 1.0	< 1.0	< 1.0
	t5	< 1.0	< 1.0	< 1.0	< 1.0	< 1.0	< 1.0	< 1.0	< 1.0
	t6	< 1.0	< 1.0	< 1.0	< 1.0	< 1.0	< 1.0	< 1.0	< 1.0
Mesophilic aerobic bacteria (Log CFU mL^{-1})		W1	W2	W3	W4	A1	A2	A3	A4
	t0	< 1.0	< 1.0	< 1.0	< 1.0	< 1.0	< 1.0	< 1.0	< 1.0
	t1	< 1.0	< 1.0	< 1.0	< 1.0	< 1.0	< 1.0	< 1.0	< 1.0
	t2	2.48 ± 25	< 1.0	< 1.0	< 1.0	< 1.0	< 1.0	< 1.0	< 1.0
	t3	2.46 ± 23	< 1.0	< 1.0	< 1.0	< 1.0	< 1.0	< 1.0	< 1.0
	t4	2.89 ± 2.54	< 1.0	< 1.0	< 1.0	< 1.0	< 1.0	< 1.0	< 1.0
	t5	4.04 ± 06	< 1.0	< 1.0	< 1.0	2.67 ± 0.06	< 1.0	< 1.0	< 1.0
	t6	4.69 ± 03	< 1.0	< 1.0	< 1.0	4.61 ± 0.05	< 1.0	< 1.0	< 1.0
Yeasts (Log CFU mL^{-1})		W1	W2	W3	W4	A1	A2	A3	A4
	t0	< 1.0	< 1.0	< 1.0	< 1.0	< 1.0	< 1.0	< 1.0	< 1.0
	t1	< 1.0	< 1.0	< 1.0	< 1.0	< 1.0	< 1.0	< 1.0	< 1.0
	t2	< 1.0	< 1.0	< 1.0	< 1.0	< 1.0	< 1.0	< 1.0	< 1.0
	t3	3.48 ± 02	< 1.0	< 1.0	< 1.0	18 ± 2.50	< 1.0	< 1.0	< 1.0
	t4	3.59 ± 00	< 1.0	< 1.0	< 1.0	1.72 ± 2.44	< 1.0	< 1.0	< 1.0
	t5	3.47 ± 0.07	< 1.0	< 1.0	< 1.0	3.52 ± 0.04	< 1.0	< 1.0	< 1.0
	t6	3.56 ± 0	< 1.0	< 1.0	< 1.0	3.61 ±0.05	< 1.0	< 1.0	< 1.0
Enterobacteriaceae (Log CFU mL^{-1})		W1	W2	W3	W4	A1	A2	A3	A4
	t0	< 1.0	< 1.0	< 1.0	< 1.0	< 1.0	< 1.0	< 1.0	< 1.0
	t1	< 1.0	< 1.0	< 1.0	< 1.0	< 1.0	< 1.0	< 1.0	< 1.0
	t2	< 1.0	< 1.0	< 1.0	< 1.0	< 1.0	< 1.0	< 1.0	< 1.0
	t3	< 1.0	< 1.0	< 1.0	< 1.0	< 1.0	< 1.0	< 1.0	< 1.0
	t4	< 1.0	< 1.0	< 1.0	< 1.0	< 1.0	< 1.0	< 1.0	< 1.0
	t5	< 1.0	< 1.0	< 1.0	< 1.0	< 1.0	< 1.0	< 1.0	< 1.0
	t6	< 1.0	< 1.0	< 1.0	< 1.0	< 1.0	< 1.0	< 1.0	< 1.0

Microbial counting of pickled sea fennel in vinegar Batch 2.

Microbiological group	Sampling (t,months)	Prototypes							
Mesophilic lactobacilli (Log CFU mL^{-1})		W1	W2	W3	W4	A1	A2	A3	A4
	t0	< 1.0	< 1.0	< 1.0	< 1.0	< 1.0	< 1.0	< 1.0	< 1.0
	t1	< 1.0	< 1.0	< 1.0	< 1.0	< 1.0	2.13 ± 3.01	< 1.0	< 1.0
	t2	< 1.0	< 1.0	< 1.0	< 1.0	< 1.0	1.56 ± 2.21	< 1.0	< 1.0
	t3	< 1.0	< 1.0	< 1.0	< 1.0	< 1.0	1.54 ± 2.17	< 1.0	< 1.0

	t4	< 1.0	< 1.0	< 1.0	< 1.0	< 1.0	1.54 ± 2.17	< 1.0	< 1.0
	t5	< 1.0	< 1.0	< 1.0	< 1.0	< 1.0	0.85 ± 1.20	< 1.0	< 1.0
	t6	< 1.0	< 1.0	< 1.0	< 1.0	< 1.0	< 1.0	< 1.0	< 1.0
Mesophilic aerobic bacteria (Log CFU mL^{-1})		W1	W2	W3	W4	A1	A2	A3	A4
	t0	< 1.0	< 1.0	< 1.0	< 1.0	< 1.0	< 1.0	< 1.0	< 1.0
	t1	< 1.0	< 1.0	< 1.0	< 1.0	< 1.0	2,14 ± 3,03	< 1.0	< 1.0
	t2	< 1.0	< 1.0	< 1.0	< 1.0	< 1.0	1,52 ± 2,15	< 1.0	< 1.0
	t3	< 1.0	< 1.0	< 1.0	< 1.0	< 1.0	1,54 ± 2,18	< 1.0	< 1.0
	t4	< 1.0	< 1.0	< 1.0	< 1.0	< 1.0	1,55 ± 2.19	< 1.0	< 1.0
	t5	1.29 ± 1.82	< 1.0	< 1.0	< 1.0	2.67 ± 0.06	1,25 ± 1,76	< 1.0	< 1.0
	t6	< 1.0	< 1.0	< 1.0	< 1.0	4.61 ± 0.05	< 1.0	< 1.0	< 1.0
Yeasts (Log CFU mL^{-1})		W1	W2	W3	W4	A1	A2	A3	A4
	t0	< 1.0	< 1.0	< 1.0	< 1.0	< 1.0	< 1.0	< 1.0	< 1.0
	t1	1,36 ± 1,93	< 1.0	< 1.0	< 1.0	< 1.0	1,87 ± 2,64	< 1.0	< 1.0
	t2	1,87 ± 2,64	< 1.0	< 1.0	< 1.0	< 1.0	1,18 ± 1,67	< 1.0	< 1.0
	t3	1,25 ± 1,76	< 1.0	< 1.0	< 1.0	1.18 ± 2.50	1,00 ± 1,41	< 1.0	< 1.0
	t4	1,24 ± 1,75	< 1.0	< 1.0	< 1.0	1.72 ± 2.44	2,42 ± 3,42	< 1.0	< 1.0
	t5	1,21± 1,72	1,42 ± 2,01	< 1.0	< 1.0	3.52 ± 0.04	1,52 ±2,16	< 1.0	< 1.0
	t6	0,98 ± 1,38	< 1.0	< 1.0	< 1.0	3.61 ± 0.05	0,76 ±1,07	< 1.0	< 1.0
Enterobacteriaceae (Log CFU mL^{-1})		W1	W2	W3	W4	A1	A2	A3	A4
	t0	< 1.0	< 1.0	< 1.0	< 1.0	< 1.0	< 1.0	< 1.0	< 1.0
	t1	< 1.0	< 1.0	< 1.0	< 1.0	< 1.0	< 1.0	< 1.0	< 1.0
	t2	< 1.0	< 1.0	< 1.0	< 1.0	< 1.0	< 1.0	< 1.0	< 1.0
	t3	< 1.0	< 1.0	< 1.0	< 1.0	< 1.0	< 1.0	< 1.0	< 1.0
	t4	< 1.0	< 1.0	< 1.0	< 1.0	< 1.0	< 1.0	< 1.0	< 1.0
	t5	< 1.0	< 1.0	< 1.0	< 1.0	< 1.0	< 1.0	< 1.0	< 1.0
	t6	< 1.0	< 1.0	< 1.0	< 1.0	< 1.0	< 1.0	< 1.0	< 1.0

Color measurement

The color parameters of sea fennel pickles from batches 1 and 2 were measured at three sampling times: immediately after preparation (t_0), after 3 months (t_3), and after 6 months of storage (t_6). The measured parameters included L* (lightness), a* (red-green coordinate), b* (yellow-blue coordinate), hue angle (h°), and chroma (C), which collectively describe the color characteristics of the samples.

For Batch 1, lightness (L*) generally showed a slight increase over time in most prototypes, indicating that the pickles became somewhat lighter during storage. In more detail, the sample W2 showed an increase of L* from 32.25 at t0 to 37.24 at t6. The a* values remained consistently negative across all prototypes and times, indicating a stable greenish hue that did not significantly change with storage. The b* values, representing yellowness, tended to decrease over time in most samples, suggesting a reduction in yellow coloration as storage progressed. Hue angle (h°) remained relatively stable across time points, indicating that the overall color tone did not significantly change. Chroma (C), which reflects color saturation, generally decreased over storage, suggesting a loss of color intensity or dulling of the samples.

In Batch 2, a similar pattern was observed, with some differences in the magnitude of changes. Lightness (L*) generally increased from t_0 to t_3 and then slightly decreased or stabilized by t_6. For instance, W2 increased from 35.08 at t_0 to 42.12 at t_3, then decreased to 38.17 at t6. The a* values remained negative, showing a persistent greenish color, although some fluctuations were noted. The b* values varied but tended to decrease over time in some prototypes, indicating a slight

reduction in yellowness. Hue angles were relatively stable, and chroma showed a moderate decrease in some samples, reflecting a slight loss in color vitality during storage.
Overall, the results suggest that the pickles undergo gradual lightning and slight loss of color saturation over six months, while the green hue remains stable. Differences between batches and prototypes were observed but were generally minor. The color changes might be influenced by factors such as acidity level, vinegar type, and storage conditions. These findings are important for understanding the visual quality and consumer appeal of sea fennel pickles during shelf life.

Color analyses of pickled sea fennel in vinegar Batch 1 and 2.

Batch	Prototype	Sampling time (t, months)	Color Parameter				
			L	a*	b*	h°	C
B1	W2						
		t0	32.25 ± 2.20^{a}	-2.49 ± 0.04^{a}	14.89 ± 1.10^{a}	103.26 ± 0.72^{a}	17.70 ± 1.08^{a}
		t3	34.79 ± 1.47^{ab}	-3.03 ± 1.07^{a}	11.92 ± 3.21^{a}	104.08 ± 1.95^{a}	12.30 ± 3.36^{a}
		t6	37.24 ± 1.66^{ab}	-2.19 ± 0.43^{a}	10.17 ± 1.17^{b}	102.18 ± 2.36^{a}	10.41 ± 1.18^{b}
	W3						
		t0	36.34 ± 3.03^{a}	-3.27 ± 1.46^{a}	17.79 ± 3.36^{a}	100.08 ± 2.64^{a}	18.10 ± 3.57^{a}
		t3	34.35 ± 0.81^{ab}	-2.04 ± 0.25^{a}	8.23 ± 0.46^{a}	103.99 ± 2.38^{a}	8.49 ± 0.39^{a}
		t6	37.02 ± 3.06^{ab}	-2.23 ± 0.50^{a}	9.10 ± 1.06^{b}	103.67 ± 1.61^{a}	9.37 ± 1.15^{b}
	W4						
		t0	35.43 ± 1.90^{a}	-3.14 ± 0.96^{a}	15.58 ± 3.59^{a}	101.27 ± 1.61^{a}	15.90 ± 3.69^{a}
		t3	36.20 ± 1.21^{a}	-2.37 ± 0.89^{a}	9.59 ± 2.50^{a}	103.58 ± 2.34^{a}	9.88 ± 2.63^{a}
		t6	33.86 ± 1.50^{b}	-2.75 ± 0.84^{a}	8.91 ± 2.04^{b}	106.92 ± 2.06^{a}	9.33 ± 2.19^{b}
	A2						
		t0	33.51 ± 1.31^{a}	-2.77 ± 0.13^{a}	13.31 ± 1.88^{a}	101.95 ±2.30^{a}	13.60 ± 1.81^{a}
		t3	34.27 ± 2.12^{ab}	-2.67 ± 0.90^{a}	12.30 ± 3.50^{a}	102.15 ± 0.74^{a}	12.59 ± 3.61^{a}
		t6	40.76 ± 1.96^{a}	-2.89 ± 1.59^{a}	14.34 ± 1.42^{a}	101.12 ± 5.02^{a}	14.65 ± 1.70^{a}
	A3						
		t0	33.29 ± 1.92^{a}	-2.36 ± 1.45^{a}	13.66 ± 2.32^{a}	99.27 ± 4.77^{a}	13.89 ± 2.51^{a}
		t3	33.31 ± 0.38^{ab}	-1.82 ± 0.60^{a}	7.85 ± 1.27^{a}	102.77 ± 2.45^{a}	8.06 ± 1.36^{a}
		t6	35.16 ± 2.06^{ab}	-2.65 ± 0.17^{a}	9.26 ± 0.85^{b}	105.99 ± 0.55^{a}	9.63 ± 0.86^{b}
	A4						
		t0	33.72 ± 1.48^{a}	-3.52 ± 0.88^{a}	14.95 ± 2.67^{a}	103.16 ± 0.99^{a}	15.36 ± 2.79^{a}
		t3	32.33 ± 0.88^{b}	-1.83 ± 0.49^{a}	8.89 ± 2.00^{a}	101.78 ± 2.75^{a}	9.08 ± 2.00^{a}
		t6	33.33 ± 0.79^{b}	-1.60 ± 0.09^{a}	7.32 ± 0.38^{b}	102.32 ± 0.12^{a}	7.49 ± 0.39^{b}
B2	W2						
		t0	35.08 ± 0.36^{b}	-5.48 ± 1.58^{a}	19.29 ± 2.21^{a}	105.70 ± 2.64^{a}	20.06 ± 2.56^{a}
		t3	42.12 ± 1.70^{a}	-3.40 ± 1.07^{a}	17.71 ± 1.22^{a}	100.85 ± 0.83^{a}	18.03 ± 1.29^{a}
		t6	38.17 ± 0.55^{b}	-4.56 ± 0.02^{a}	15.79 ± 0.04^{a}	106.10 ± 0.11^{a}	16.43 ± 0.03^{b}
	W3						
		t0	35.80 ± 0.96^{b}	-5.55 ± 0.05^{a}	17.20 ± 0.59^{a}	107.88 ± 0.73^{a}	18.07 ± 0.55^{a}
		t3	38.91 ± 0.07^{a}	-4.28 ± 0.01^{a}	17.56 ± 3.13^{a}	103.90 ± 2.43^{a}	18.08 ± 3.04^{a}
		t6	36.03 ± 1.45^{ab}	-2.95 ± 1.10^{a}	14.06 ± 1.94^{a}	101.63 ± 2.72^{a}	14.37 ± 2.13^{b}
	W4						
		t0	37.37 ± 1.88^{a}	-3.40 ± 0.13^{a}	14.34± 0.04^{a}	103.34 ± 0.04^{a}	13.34 ± 0.47^{a}
		t3	36.20 ± 1.21^{a}	-2.37 ± 0.89^{b}	9.59 ± 2.50^{b}	103.58 ± 2.34^{a}	9.88 ± 2.63^{b}
		t6	35.12 ± 2.91^{a}	-3.20 ± 0.59^{b}	13.46 ± 1.12^{b}	103.50 ± 3.44^{a}	13.84 ± 0.95^{b}
	A2						
		t0	38.26 ± 1.50^{a}	-5.71 ± 0.14^{a}	20.44± 0.24^{a}	105.62 ±0.55^{a}	21.22 ± 0.19^{a}
		t3	40.07 ± 2.56^{a}	-3.30 ± 0.50^{b}	12.98± 1.09^{b}	104.35 ± 3.23^{a}	13.40 ± 0.93^{b}
		t6	32.75 ± 0.94^{a}	-3.51 ± 0.66^{b}	14.90 ± 2.82^{ab}	103.25 ± 0.01^{a}	15.30 ± 2.90^{ab}
	A3						
		t0	32.24 ± 0.63^{a}	-3.65 ± 0.29^{a}	14.70 ± 0.44^{a}	103.95 ± 1.47^{a}	15.15 ± 0.36^{a}
		t3	37.66 ± 1.06^{a}	-3.41 ± 0.94^{a}	14.13 ± 1.33^{a}	103.45 ± 2.40^{a}	14.54 ± 1.51^{a}
		t6	37.04 ± 2.61^{a}	-4.10 ± 0.12^{a}	15.35 ± 1.18^{a}	105.00 ± 1.54^{a}	15.89 ± 1.11^{a}
	A4						
		t0	35.82 ± 2.29^{a}	-4.43 ± 1.38^{a}	16.67 ± 3.28^{a}	104.70 ± 1.68^{a}	17.25 ± 2.79^{a}

t3	37.12 ± 0.07[a]	-3.44 ± 0.30[a]	14.37 ± 0.27[a]	103.46 ± 1.38[a]	14.78 ± 0.19[a]
t6	36.27 ± 2.13[a]	-3.22 ± 0.98[a]	13.42 ± 2.10[a]	103.33 ± 1.96[a]	13.81 ± 2.27[a]

The results are expressed as the means of three replicates (± standard deviation). Within each row, for the same batch and each color parameter, overall means marked with different lowercase superscript letters are significantly different ($p < 0.05$).

Salt measurement

A general decrease in salt content was observed across the monitoring period from month 0 to month 6 in both batches. Initially, the salt content was close to the residual salt in the fermented sea fennel after washing and before pasteurization (3%), with minor differences likely attributed to the application of vinegar and varying acidity levels. Regarding the type of vinegar used, no significant differences were noted between wine and apple vinegar. No differences were observed between the different acidity levels applied as salt concentration decreased in all samples over time.

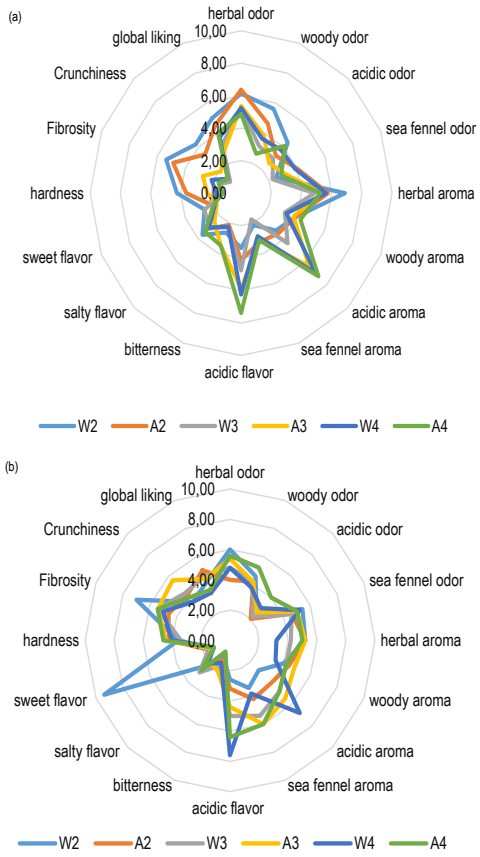
Salt Content in Pickles with Sea Fennel in Batches 1 and 2 at Months 0 and 6.

Sampling time (t, months)	Sample	Batch1	Batch2
	W2	2,62 ± 0,12[a]	2,45 ± 0,57[a]
T_0	W3	2,25 ± 0,28[a]	2,15 ± 0,21[a]
	W4	2,43 ± 0,28[a]	2,23 ± 0,28[a]
	A2	2,05 ± 0,26[a]	2,10 ± 0,00[a]
	A3	2,68 ± 0,06[a]	2,33 ± 0,28°
	A4	2,61 ± 0,32[a]	2,33 ± 0,40[a]
T_6			
	W2	1,05 ± 0,00[b]	2,00 ± 0,00[a]
	W3	1,65 ± 0,13[b]	1,20 ± 0,07[b]
	W4	1,48 ± 0,08[b]	1,45 ± 0,07[b]
	A2	1,32 ± 0,06[b]	1,20 ± 0,00[b]
	A3	1,70 ± 0,28[b]	1,28 ± 0,11[b]
	A4	1,70 ± 0,21[b]	1,28 ±0,18[b]

The results are expressed as the means of three replicates (± standard deviation). Within each row, for the same, overall means marked with different lowercase superscript letters are significantly different ($p < 0.05$).

Sensory analyses

The sensory analysis of pickles made with sea fennel showed that Batch 2 maintained better textural stability, with higher scores for hardness, fibrosity, and crunchiness, compared to Batch 1. This difference in stability may be attributed to environmental factors, as Batch 1 was produced in summer 2023 and stored at room temperature, while Batch 2 was made during the cooler winter months of 2024. In more detail, an increase in acidity led to a decline in herbal and woody odors and aromas, suggesting that higher acidity levels may suppress these characteristics. Notably, sea fennel odor and aroma were most highly detected in samples with 0.5% acidity, regardless of the type of vinegar used, indicating that moderate acidity enhances these sensory attributes. On the other hand, acidic aroma and flavor intensified with higher acidity levels, negatively affecting overall acceptability due to the perception of an overly acidic flavor and textural degradation. Preference trends also differed by vinegar type, with wine vinegar at 0.5% acidity being favored in Batch 1 and apple vinegar in Batch 2. However, type of vinegar did not highly affect the individual sensory attributes These findings suggest that moderate acidity (0.5%) provides a balance that preserves sensory quality and highlights the characteristic profile of sea fennel.



Results of sensory analysis performed on pickles made with sea fennel. (a) Sensory analyses performed after 6 months: Batch 1; (b) sensory analyses performed after 6 months: Batch2.