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Exploring the potential of deep eutectic systems for the preservation of the chemical profile and antibacterial potential of dill (*Anethum graveolens* L.) supercritical CO_2 extracts

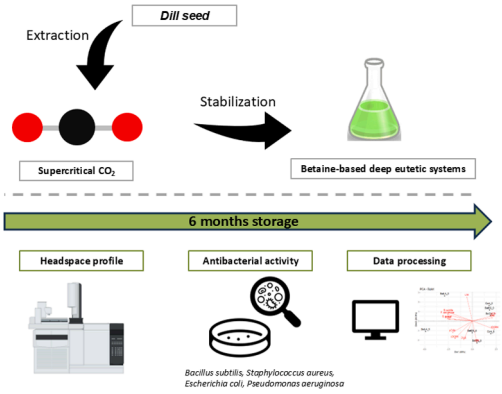
Jelena Vladić [a], Igor Jerković [b,*], Valentina Pavić [c], Dragoljub Cvetković [d], Stela Jokić [c], Krunoslav Aladić [c], Kristian Pastor [d], Ana Rita C. Duarte [a,*]

[a] LAQV-REQUIMTE, Chemistry Department, NOVA – School of Science and Technology, Caparica 2829-516, Portugal
[b] Faculty of Chemistry and Technology, University of Split, Split 21000, Croatia
[c] Faculty of Food Technology Osijek, University of Josip Juraj Strossmayer in Osijek, Osijek 31000, Croatia
[d] Faculty of Technology Novi Sad, University of Novi Sad, Novi Sad 21000, Serbia

HIGHLIGHTS

- Supercritical CO_2 and DESs combined to extract and stabilize dill volatiles.
- DESs effectively preserved the chemical profile of dill extract during storage.
- Dill-Bet/LA system showed enhanced antibacterial activity versus the dill control.
- Dill-Bet/LA system best preserved the antimicrobial potency during storage.

GRAPHICAL ABSTRACT



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ABSTRACT

This study evaluated the potential of deep eutectic systems (DESs) to enhance the chemical and microbiological stability, as well as the antibacterial activity of dill (*Anethum graveolens* L.) supercritical extracts, rich in carvone and limonene, over six months of storage. Various betaine-based DESs were tested for their ability to preserve the chemical integrity and enhance the antibacterial properties of dill extracts. Significant alterations in the headspace profile of extracts during storage indicated degradation reactions. The dill-Bet/LA extract system (dill extract in Bet/LA) proved most effective, significantly slowing degradation and enhancing antibacterial activity

Abbreviations: AcOH, Acetic acid; HA, Hexanal; αPIN, *α*-Pinene; SAB, Sabinene; βMYC, *β*-Myrcene; αPHLD, *α*-Phellandrene; PCYM, *p*-Cymene; LIM, Limonene; PCYMN, *p*-Cymenene; CMNT, *cis-p*-Menth-2,8-dien-1-ol; CLIMO, *cis*-Limonene oxide; TLIMO, *trans*-Limonene oxide; DILE, Dill ether; αTRL, *α*-Terpineol; CDCRN, *cis*-Dihydrocarvone; TDCRN, *trans*-Dihydrocarvone; IDCRL, Isodihydrocarveol; CCRL, *cis*-Carveol; TCRL, *trans*-Carveol; CRN, Carvone; TCRNO, *trans*-Carvone oxide; ANE, Anethol; LIMOH, Limonene-1,2-diol; CRNEP, Carvone-7,8-epoxide; CRNEP*, Carvone-7,8-epoxide*; MYSN, Myristicin; DLP, Dillapiole.

* Corresponding authors.
E-mail addresses: igor@ktf-split.hr (I. Jerković), ard08968@fct.unl.pt (A.R. C. Duarte).

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compared to the dill-control extract. Specifically, this system exhibited 20–40-fold greater efficacy against Gram-negative bacteria and 5–8-fold stronger activity against Gram-positive bacteria than the dill-control. Furthermore, the dill-Bet/LA system was also the most effective in preserving antimicrobial potency during storage, demonstrating its potential as a sustainable solution for extending the shelf life and bioactivity of dill supercritical extracts.

1. Introduction

Dill (*Anethum graveolens* L.), a member of the *Apiaceae* family and the genus Anethum, is a globally important aromatic plant used as a spice for seasoning and flavoring a wide array of foods, including rice, sauces, salads, side dishes, and soups. Its essential oil, noted for its distinctive aromatic and herbaceous qualities, is extensively used in the food industry as a flavoring agent. Additionally, the oil's aromatic properties make it valuable in the production of personal care and cleaning products, such as soaps and detergents [1]. Thanks to its rich content of bioactive aromatic compounds, particularly carvone and limonene [2], dill oil also finds significant applications in pharmaceuticals, skincare, cosmetics, and pest control products [3]. Carvone, the dominant compound in dill oil, exhibits antimicrobial and antifungal properties, making it essential for food preservation and pharmaceutical formulations. Limonene, another major constituent, is recognized for its antioxidant and antimicrobial effects, which complement the activity of carvone [4]. Additionally, minor compounds like α-phellandrene enhance the therapeutic and preservative potential of dill extracts, further broadening their versatility [5].

The diverse applications and increasing demand for dill and dill oil products underscore the economic importance of this plant species. According to a VMR (Verified Market Reports) report, the dill seed oil market was valued at USD 1.57 billion in 2023 and is projected to reach USD 2.18 billion by 2030, with a compound annual growth rate of 4.29 % during the forecast period from 2024 to 2030 [6]. This growing demand justifies accelerated product development and the adoption of advanced extraction and stabilization technologies.

Traditionally, dill products have been obtained using methods such as hydrodistillation, steam distillation, and organic solvent extraction [7–9]. However, advanced technologies, such as supercritical CO_2 extraction, have been investigated [10] and shown to offer advantages over traditional methods [11]. Supercritical CO_2 extraction provides several benefits, including environmental friendliness, low cost, non-flammability, recyclability, and the ability to fine-tune and adjust extraction conditions to obtain target components and achieve higher yields. Additionally, unlike conventional techniques for extracting lipid-based components, such as Soxhlet extraction, where the organic solvent is removed by evaporation but may still leave trace residues, supercritical CO_2 is fully removed from the sample simply by reducing the pressure, resulting in a pure and safe extract [12].

Previous research has shown that supercritical CO_2 extraction can significantly enhance the yield and quality of dill extracts. For example, Li et al. [10] optimized extraction conditions and identified carvone and limonene as major compounds, demonstrating supercritical CO_2 ability to produce high yields with superior selectivity. Similarly, Nautiyal and Tiwari [13] found that supercritical CO_2 extraction produced higher concentrations of key bioactive compounds like carvone and limonene compared to hydrodistillation, while also reducing process time and energy consumption. Garcez et al. [11] further validated the efficiency of supercritical CO_2 extraction by comparing it with other techniques and highlighting its ability to recover bioactive compounds such as carvone and dillapiole in a cleaner and more targeted manner.

An emerging alternative to traditional solvents is deep eutectic solvents (DESs), which are characterized by easy preparation, low cost, biodegradability, and low or non-toxicity [14]. These alternative solvents are formed by mixing two or more components, typically a hydrogen bond donor and a hydrogen bond acceptor, to create a eutectic mixture with a melting point significantly lower than that of the individual components. DESs not only have notable extraction capabilities but also exhibit stabilization properties for bioactive components [15, 16]. The strong hydrogen bonding interactions within DESs can protect sensitive compounds from degradation, enhancing their stability [17]. Previous studies have indicated that DESs have the potential to stabilize volatile aroma compounds and consequently increase the shelf life of products. For instance, the use of DES extended the shelf life of lavender and rosemary extracts, which are rich in volatile aroma components [18, 19]. In contrast, control samples without DES underwent significant changes within six months of storage at room temperature in the dark, indicating decreased stability. However, previous studies did not address the microbiological stability of the samples or changes in their bioactivity.

This study aims to fill these gaps by investigating, for the first time, the capacity of different DESs to preserve the antibacterial activity of dill volatile components extracted using supercritical fluid extraction. Additionally, to determine the correlation between volatile organic compounds and bioactivity over extended periods, the chemical profile of the extracts was monitored using headspace analysis, along with the microbiological status of the dill samples. The stability of dill aroma volatile compounds was examined during storage at room temperature and under light exposure.

By combining eco-friendly solvents, such as supercritical CO_2 and DESs, with detailed chemical and microbiological analyses, this study aims to provide valuable insights into the long-term stability and safety of dill bioactive compounds with a reduced environmental footprint. Moreover, improving the stability and efficacy of dill extracts through these methods can enhance the availability and quality of dill products, thereby supporting sustainable development in the food, agricultural, and pharmaceutical industries.

2. Materials and methods

2.1. Sample material and chemicals

Dill fruit (*Anethum graveolens* L., Apiaceae) was purchased in 2022 from the Institute for Medicinal Plants Research 'Dr. Josif Pančić' in Belgrade, Serbia. Prior to extraction, the sample was ground using a Bosch electric grinder (Model TSM6A013B, Gerlingen, Germany). The mean particle size of the ground material was determined to be 0.235 mm using vibration sieve set from CISA (Cedaceria, Spain).

Betaine (CAS number: 107–43–7; ≥97 % purity) and levulinic acid (CAS number: 123–76–2; 98 %) were obtained from Sigma-Aldrich (St. Louis, MO, USA). Glycerol (CAS number: 56–81–5; 99.5 % purity) was purchased from Scharlau (Barcelona, Spain), and ethylene glycol (CAS number: 107–21–1; ≥99.5 % purity) was acquired from Carlo Erba (Val-de-Reuil, France).

2.2. Preparation and characterization of deep eutectic systems (DESs)

Deep eutectic systems (DESs) were prepared by mixing the components in the appropriate molar ratios and then heating to 40°C while stirring until a clear liquid was formed. The molar ratios for the DESs were as follows: betaine/glycerol (Bet/Gly) 1:2, betaine/ethylene glycol (Bet/EG) 1:3, and betaine/levulinic acid (Bet/LA) 1:2. DES mixtures were selected based on previous research, and all systems used in this study had been previously characterized [19,20].

2.3. Supercritical carbon dioxide (CO_2) extraction and dispersion in DES

Supercritical extraction was conducted using a high-pressure extraction system (HPEP, NOVA-Swiss, Effretikon, Switzerland) with the following main specifications: a CO_2 gas cylinder, a diaphragm-type compressor (with a pressure range up to 1000 bar), an extractor vessel with a heating jacket (internal volume 200 mL, maximum operating pressure 700 bar), a separator with a cooling jacket (internal volume 200 mL, maximum operating pressure 250 bar), a pressure control valve, a temperature regulation system, and regulation valves. Extractions were carried out under the following conditions: 80 bar pressure, 40 °C temperature, CO_2 flow rate of 0.194 kg/h, and an extraction time of 4 hours. Extraction was performed in triplicate, yielding 3.98 % (w/w). The dispersion of the extract in DES was conducted following the process described in previous work (Vladic et al., 2023). After 4 hours of extraction, depressurization was applied, and the extract was dispersed in the prepared DES. The mixture was then homogenized using a vortex mixer for 1 minute. The ratio of DES to extract was 0.05 g ± 10 % extract per 1 mL DES, chosen to ensure easy homogenization.

The control (dill-control) and CO_2 extracts dispersed in DES (dill-DES) were kept in transparent containers at room temperature (27°C ± 3°C) under light/dark cycle conditions. The chemical profile of dill-control and dill-DES extracts was analyzed at the beginning (0 months), after 3 months, and after 6 months of storage. Microbiological stability and antibacterial activity were analyzed at the beginning (0 months) and after 6 months of storage.

2.4. Headspace solid-phase microextraction (HS-SPME) and gas chromatography with mass spectrometry analysis (GC–MS)

Headspace volatiles were extracted using a manual solid-phase microextraction (SPME) fiber coated with divinylbenzene/carbon wide range/polydimethylsiloxane (DVB/Carbon WR/PDMS) from Supelco Co. (Bellefonte, PA, USA). The fiber was conditioned as per the manufacturer's instructions. For HS-SPME, 1.5 g of the sample was placed in a 15-mL glass vial, which was hermetically sealed with PTFE/silicone septa. The vial was placed in a water bath at 60°C for 15 minutes to equilibrate, followed by a 45-minute extraction period for HS-SPME. After sampling, the SPME fiber was retracted into the needle, removed from the vial, and inserted into the injector (250°C) of the gas chromatography–mass spectrometry (GC–MS) system for 6 minutes of thermal desorption directly into the GC column.

The prepared samples were analyzed using an Agilent 7890B gas chromatograph coupled to a mass spectrometer Agilent, series 5977 A (Agilent Technologies, Palo Alto, CA, USA). The components were separated on an HP-5MS capillary column (30 m × 0.25 mm, 0.25 μm, Agilent Technologies). The injector temperature was set at 250°C in split mode (1:50), with helium as the carrier gas at a constant flow of 1 mL/min. The GC temperature program started at 70°C for 2 minutes, increased at a rate of 3 °C/min to 200°C, and then held at 200°C for 15 minutes. The mass spectrometer operated at 70 eV in scan mode with an m/z range of 30–300. The injector and detector temperatures were set at 250°C and 300°C, respectively.

Qualitative identification of the compounds was achieved using the Wiley 9 (Wiley, New York, NY, USA) and NIST 17 (National Institute of Standards and Technology, Gaithersburg, MD, USA) mass spectral libraries, along with literature data on retention indices calculated with C_9–C_{25} alkanes. Each sample was analyzed in triplicate, and the results are presented as mean values.

2.5. Assessment of microbiological stability

Microbiological determination of total aerobic microbial count, and total molds and yeasts count were performed according to the existing ISO Standard Microbiological Methods [21,22]. Spores of anaerobic mesophilic bacteria (SAMB) were determined on Nutrient Agar (HiMedia, Mumbai, India) incubated under anaerobic conditions at 30 ± 1°C for 72 ± 3 hours after a 5-minute dip sample in boiling water. After incubation at appropriate temperatures, the colonies that appeared on the selected plates were counted as colony-forming units (cfu) per mL of sample. Each test on all samples was performed in triplicate. Results were expressed as mean values.

2.6. Assessment of antibacterial activity

The minimal inhibitory concentrations (MIC) of the obtained extracts were determined using a modified broth microdilution method in Mueller Hinton Broth (MHB) (Fluka, BioChemica, Germany) following the Clinical Laboratory and Standards Institute (CLSI) M7-A7 guidelines [23]. Four human pathogens, representing both Gram-positive and Gram-negative bacteria, were tested: *Bacillus subtilis, Staphylococcus aureus, Escherichia coli,* and *Pseudomonas aeruginosa*. These strains were isolated from various clinical specimens provided by the Microbiology Service of the Public Health Institute of Osijek-Baranja County, Croatia.

Mid-logarithmic-phase bacterial cultures (5 × 10^5 CFU/mL) in MHB were added to two-fold serial dilutions of the extracts (50–0.00122 mg/mL), which were prepared using broth as the diluent. Each plate included a growth control (bacterial inoculum without extracts) and a negative control (broth and ethanol). Ciprofloxacin (Hospira, Hurley, Maidenhead, Berkshire, England, UK) served as the antibacterial standard, tested at concentrations ranging from 0.122 to 250 μg/mL. Following a 24-hour incubation at 37°C, an additional 3-hour incubation with triphenyl tetrazolium chloride was conducted to indicate microbial growth. MIC values were derived from triplicate analyses, normalized against the negative control, and expressed in milligram of extract per milliliter.

2.7. Data processing and visualization tools

Heat-map clustering was employed as a powerful visualization tool to explore and intuitively interpret the volatile headspace data, highlighting patterns, relationships, and trends. The open-source platform Heatmapper (http://www.heatmapper.ca/) was used to cluster the dill-DES samples, based on volatiles composition and content determined via HS-SPME GCMS.

To assess the strength and direction of linear relationships between continuous variables (volatile composition and contents) and antibacterial activities against *Bacillus subtilis*, *Staphylococcus aureus*, *Escherichia coli*, and *Pseudomonas aeruginosa*, Pearson correlation analysis was utilized. Volatile components exhibiting moderate, strong, or very strong correlations (both positive and negative) with antibacterial activities were selected for further data treatment via principal component analysis (PCA).

PCA was applied to efficiently visualize and understand the hidden insights from the data. The input data for PCA included: (i) investigated deep eutectic systems of dill as scores; and both (ii) antibacterial activities against the four bacterial strains measured at 0 and 6 months; and (iii) contents of pivotal volatile compounds selected based on the correlation analysis, serving as loadings. When assessing antibacterial potential, reciprocal values of obtained MIC values were taken into account.

The open-source software Paleontological Statistics (PAST) v4.10 (Natural History Museum, University of Oslo, Norway) and RStudio v.2024.04.2 + 764 with the packages "corrr," "ggcorrplot," "FactoMineR," and "factoextra" were utilized for this purpose, as demonstrated by Habuš et al. and the RStudio Team [24,25].

3. Results and discussion

3.1. Identified components and their distribution in dill supercritical extracts

In the dill supercritical extracts, various components belonging to different chemical classes were identified, including monoterpene hydrocarbons, oxygenated monoterpenes, phenylpropanoids, carboxylic acids, and alkyl aldehydes. These identified components accounted for 90.31–99.57 % of the headspace profile of the samples. At the start (0 months), oxygen-containing monoterpenes and monoterpene hydrocarbons were predominantly present, with carvone representing 43.42–56.06 % and limonene representing 33.85–46.00 % of the total headspace profile (Table 1).

Due to variations in cultivation conditions, geographical location, and extraction methods, significant differences in the chemical composition of dill essential oil are reported in the literature. For instance, essential oil from dill cultivated in Serbia under varying light conditions (non-shaded and shaded plants) and obtained by hydrodistillation had a similar component distribution to our study, with carvone (46.1 %-49.8 %) and limonene (37.8 %-43.8 %) as the dominant components [2]. Jianu et al. [26] reported that essential oil from dill seed isolated by steam distillation had carvone (66.2 %) and limonene (26.79 %) as the most abundant components. Lee et al. [10] listed D-carvone (40.36 %), D-limonene (19.31 %), and apiol (17.50 %) as the most abundant components in extracts obtained with supercritical CO_2 (200 bar, 40°C, 120 min). In the essential oil of dill fruit cultivated in Thailand, the most abundant components were dillapiole (19.98 %-48.9 %), D-carvone (18.05 %-28.02 %), and D-limonene (26.96 %-44.61 %) [27]. Meanwhile, in dill essential oils from Bulgaria, the main components were carvone (33.57 %), myristicin (24.21 %), limonene (15.02 %), and dihydrocarvone (13.13 %) [28].

3.2. Changes in volatile profile during storage

Storage of dill extracts under light/dark cycles at room temperature resulted in changes in the distribution of components. Over time, the share of hydrocarbons decreased while the presence of oxygenated monoterpenes increased (Fig. 1). The dill-control sample and dill-Bet/Gly showed a significant drop in hydrocarbons presence after 3 months, which continued over time, reducing from 43.11 % to 18.67 % in the dill-control and from 37.28 % to 3.97 % in Bet/Gly. The other two dill-DES samples (dill-Bet/EG and dill-Bet/LA) also showed a decrease in hydrocarbons in the first three months. However, their representation remained approximately the same after 6 months, indicating a slowing down of the degradation of hydrocarbons in these extracts.

Changes in the total presence of hydrocarbons were primarily due to a significant decrease in limonene, the most abundant representative of monoterpene hydrocarbons. The presence of limonene in the dill-control sample decreased dramatically from 39.87 % to 1.24 % (Table 1). Among the dill-DES samples, dill-Bet/Gly exhibited the most drastic reduction, with limonene content dropping from 33.85 % to 3.76 %. Dill-Bet/EG had approximately a 2.5-fold reduction in limonene content after 6 months, while dill-Bet/LA demonstrated the greatest resistance to degradation, maintaining 28.21 % of its limonene content after 6 months compared to the initial 46 %.

Misharina et al. [29] reported similar observations with limonene in marjoram essential oil, where it significantly decreased during storage in light. Limonene is known for its instability and susceptibility to oxidation and degradation due to reactive double bonds, leading to the formation of various oxidation products. The significant drop in limonene percentage, along with the appearance and increased presence of compounds that could be its degradation products, such as limonene oxides, *cis/trans*-dihydrocarvone, carvone, and *cis-p*-menth-2, 8-dien-1-ol [30], may confirm limonene degradation. Bitterling et al. [30] reported that commercial caraway essential oil showed an initial decrease in limonene paired with an increase in carvone, followed by a

Table 1
The volatile headspace compounds (%) of *Anethum graveolens* extracts obtained by supercritical carbon dioxide (80 bar pressure, 40 °C temperature, CO_2 flow rate of 0.194 kg/h, and an extraction time of 4 hours).

Compound	RI	Dill-control			Dill-Bet/LA			Dill-Bet/Gly			Dill-Bet/EG		
		0 m	3 m	6 m	0 m	3 m	6 m	0 m	3 m	6 m	0 m	3 m	6 m
Acetic acid	< 900	-	0.49	1.21	-	-	-	-	-	-	-	-	-
Hexanal	< 900	0.02	0.01	-	-	-	-	-	-	-	-	-	-
α-Pinene	940	0.19	0.32	0.23	-	-	-	0.17	-	-	0.18	0.04	0.05
Sabinene	975	0.05	0.13	0.08	-	-	-	0.05	-	-	0.05	-	0.01
β-Myrcene	993	0.51	0.46	0.16	0.38	0.34	0.32	0.55	-	-	0.66	0.16	0.16
α-Phellandrene	1011	2.49	2.67	16.56	1.33	0.43	0.55	2.66	0.57	-	3.43	1.03	1.09
p-Cymene	1033	-	-	-	-	0.57	0.58		0.17	0.08	-	0.23	0.22
Limonene	1035	39.87	21.22	1.24	46	26.14	28.21	33.85	10.83	3.76	39.16	13.42	15.96
p-Cymenene	1094	-	-	0.4	-	-	-	-	0.28	0.13	-	0.23	0.23
cis-p-Menth−2.8-dien−1-ol	1126	-	0.24	0.36	-	0.08	-	-	0.09	0.11	-	0.06	0.05
cis-Limonene oxide	1139	-	1.24	0.83	-	0.03	-	-	0.18	-	-	0.29	0.27
trans-Limonene oxide	1144	-	0.9	1.42	-	-	-	-	-	-	-	-	0.03
Dill ether	1185	0.31	0.51	0.43	0.29	0.53	0.5	0.38	0.47	0.37	0.36	0.46	0.4
α-Terpineol	1193	-	-	-	-	-	-	-	-	0.05	-	-	0.04
cis-Dihydrocarvone	1195	0.82	2.45	3.54	2.6	4.93	4.82	1.14	3.54	3.52	1.23	3.36	2.97
trans-Dihydrocarvone	1200	3.34	7.98	6.18	0.64	1.12	1.07	4.38	3.64	2.89	3.58	4.17	3.54
Isodihydrocarveol	1218	-	-	-	-	0.09	0.13	-	0.53	0.98	-	0.06	0.47
cis-Carveol	1230	-	-	0.9	-	0.24	0.12	-	0.6	0.48	-	0.38	0.05
trans-Carveol	1230	0.18	-	0.46	-	0.12	0.31	0.22	0.47	0.46	-	0.31	0.23
Carvone	1249	51.79	55.24	54.89	43.42	56.53	54.95	56.06	64.9	80.35	50.26	66.96	64.12
trans-Carvone oxide	1286	-	0.69	1.15	-	-	-	-	0.21	0.2	-	0.21	0.19
Anethol	1291	-	0.13	0.09	-	-	0.06	-	0.15	0.13	-	0.12	0.09
Limonene−1.2-diol	1344	-	0.33	0.95	-	-	-	-		0.21	-	-	-
Carvone−7.8-epoxide* *	1410	-	0.38	0.81	-	-	-	-	-	-	-	-	-
Carvone−7.8-epoxide* *	1414	-	0.33	0.72	-	-	-	-	-	-	-	-	-
Myristicin	1523	-	0.14	0.13	-	0.09	-	-	0.61	-	-	0.31	-
Dillapiole	1626	-	0.22	0.21	-	0.25	0.17	-	3.07	0.52	-	0.84	0.55

*Dill-Bet/LA-betaine/levulinic acid; Dill-Bet/EG-betaine/ethylene glycol; Dill-Bet/Gly-betaine/glycerol. Extracts are extracted by headspace solid-phase microextraction (HS-SPME) and analyzed by gas chromatography–mass spectrometry (GC-MS) using DVB/Carbon WR/PDMS fiber.

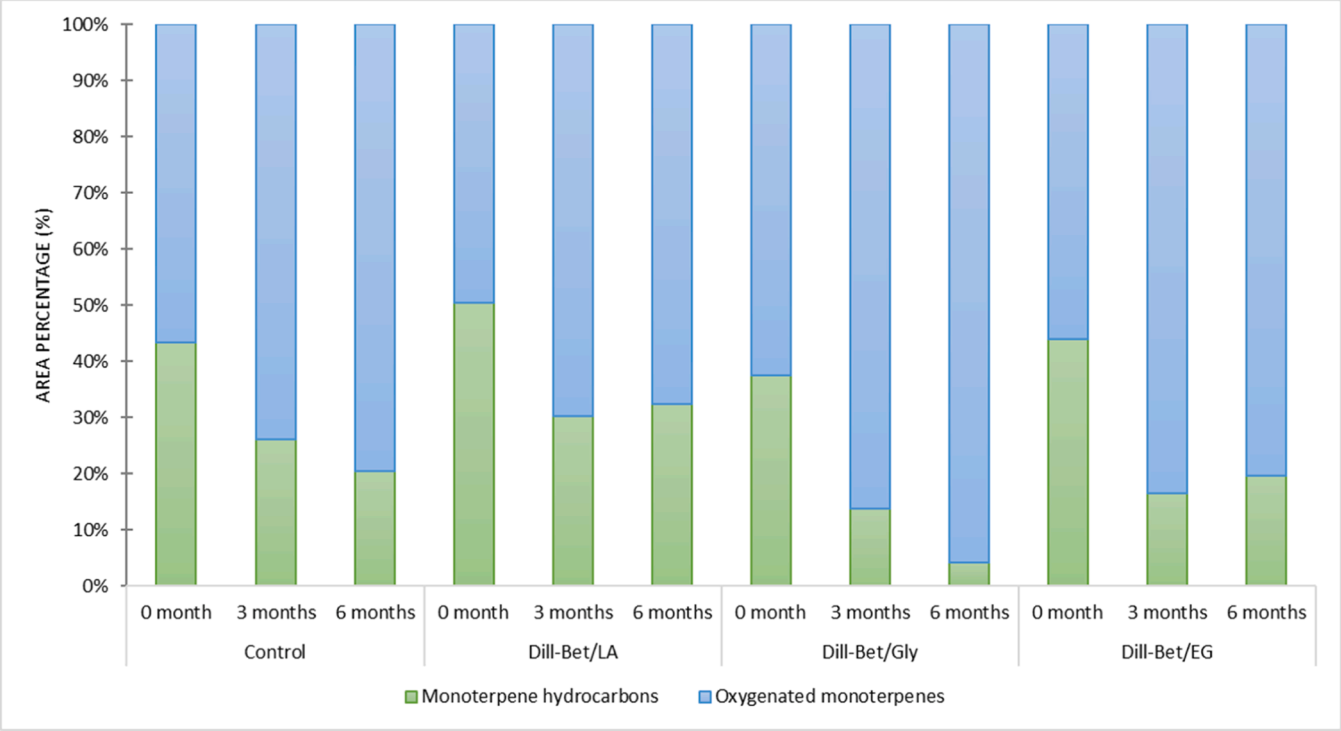

Fig. 1. Alteration of percentage distribution of monoterpene hydrocarbons and oxygenated monoterpenes in *Anethum graveolens* supercritical extracts (control and dill-DES samples) during 0, 3, and 6 months of storage under light/dark cycles and room temperature.

continued reduction in limonene with only minor changes in carvone content. This trend was also observed in the dill-control and dill-Bet/LA samples, where carvone presence rose within the first three months and then remained relatively stable, with only slight fluctuations by the six-month mark.

Interestingly, in the dill-Bet/EG and dill-Bet/Gly samples, carvone had a more substantial increase over time, with carvone rising from an initial 50.26–66.96 % and 64.12 % respectively at three and six months for dill-Bet/EG. While for dill-Bet/Gly, carvone content increased from 56.06 % initially to 80.35 % after six months. This suggests that limonene oxidation is a primary source of carvone during storage, particularly in those less stable DES samples. According to the terpene biosynthetic pathway, limonene is oxidized to *trans*-carveol via carveol as an intermediate, supporting the observed carvone formation pathway. Therefore, the formation of limonene transformation products such as carveol could also influence variations in carvone levels [30].

Additionally, degradation of limonene to limonene oxides can further produce limonene-1,2-diol, which was identified only in the dill-control sample. Initially, 11 compounds were detected in this sample (dill-control), however, after three months, a significantly higher number of newly formed components (21 compounds) was detected, indicating lower stability. Therefore, in the dill-control, limonene degradation primarily led to newly formed components rather than an increase in carvone.

Although limonene degradation products were identified in DES samples, their number was significantly lower in dill-Bet/LA sample, indicating greater stability of this system. While it is difficult to clearly establish the complex biochemical transformation processes, the detection of certain carvone oxidation products suggests that carvone may have been formed from limonene degradation and subsequently underwent further oxidation.

α-Phellandrene in dill-DES samples reduced over time, whereas the dill-control exhibited an increase in α-phellandrene percentage (16.56 %). Cătunescu et al. [31] reported an increase in α-phellandrene in minimally processed herbs, parsley, and dill, during cold storage from 1 to 8 days. Similarly, α-phellandrene in grapefruit peel oil increased with storage time (14 and 28 days) [32]. Another indicator of the low stability of the dill-control sample was the presence of acetic acid detected after 3 months (0.49 %), which increased 2.5 times after 6 months. Acetic acid was not detected in dill-DES samples.

For more effective visualization of changes during storage, a heatmap clustering is presented in Fig. 2, revealing five distinct clusters. All samples at the beginning (0 months) - dill-control (Cont 0), dill-Bet/Gly 0, dill-Bet/EG 0, and dill-Bet/LA 0 - showed similar volatile composition. A separate cluster was formed by dill-control samples after 3 and 6 months (Cont 3 and Cont 6), as well as the dill-Bet/LA samples (dill-Bet/LA 3 and dill-Bet/LA 6), indicating similar properties after 3 and 6 months. Another cluster shows dill-Bet/EG samples exhibiting similar properties after 3 and 6 months (dill-BetEG 3 and dill-BetEG 6), along with dill-Bet/Gly 3. Dill-Bet/Gly 6 showed different properties after 6 months compared to the other systems. The heatmap indicates that, apart from the most dominant constituents, limonene (LIM) shown in blue, and carvone (CRN) shown in red, contributors to the observed clustering pattern changes included α-phellandrene (αPHLD), *cis*-dihydrocarvone (cDCRN), and *trans*-dihydrocarvone (tDCRN).

The reduced alteration of the headspace profile in dill-Bet/EG and dill-Bet/LA compared to the dill-control and greater oxidative stability of these samples may be partially due to the physical protection of the components. The slowed movement of molecules due to viscosity can contribute to stabilization; however, it is not solely responsible for better stability, as Bet/Gly, the most viscous system, showed the greatest instability. The viscosity of the used systems decreases in the order Bet/Gly > Bet/LA > Bet/EG (at 30°C: Bet/Gly-1250.1 mPa.s; Bet-LA 879.93 mPa.s: Bet/EG-48.63 mPa.s) [19,20]. Therefore, viscosity is not the main determining factor of stability. It can be concluded that the composition of DES is responsible for preventing or delaying oxidative reactions and changes.

Possible explanations of stabilization mechanisms of DES could be that DES represents protective matrix for volatile compounds. This matrix reduces exposure of volatile compounds to environmental factors

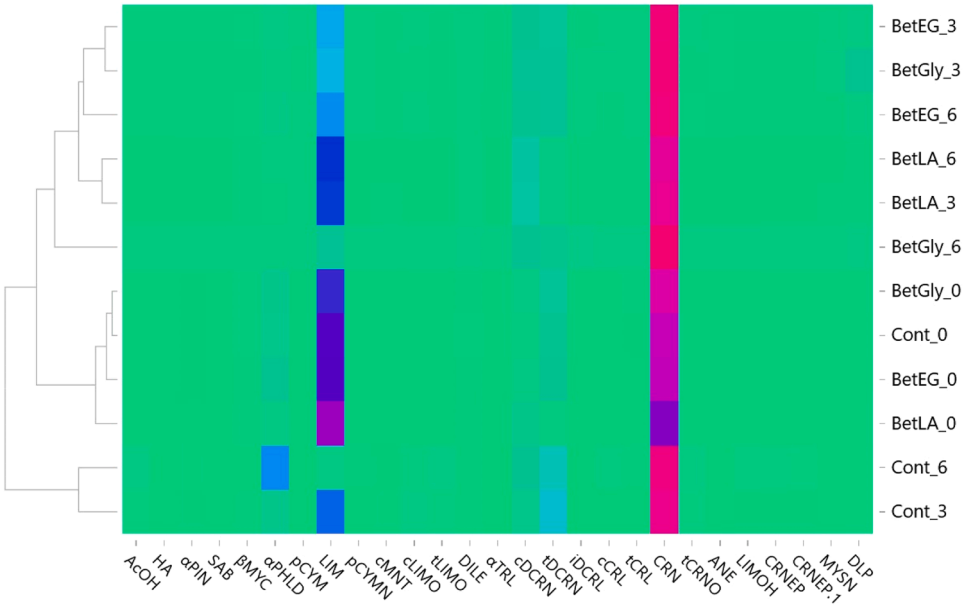

Fig. 2. Heat-map clustering of the volatile organic compounds data given in Table 1.

such as light, heat, and oxygen, which commonly trigger oxidation and volatilization. Therefore, DESs can create an effective physical barrier that limits interactions with oxidizing agents. Additionally, certain compounds present in extracts can act as hydrogen bond donors (HBDs) and hydrogen bond acceptors (HBAs) and participate in hydrogen bonding or other intermolecular forces, such as van der Waals interactions, with DES constituents. These interactions restrict the movement and reactivity of the volatile compounds, further reducing their volatilization or degradation. The low vapor pressure characteristic of DESs can also reduce the volatility of volatile compounds, thus enhancing their stability.

The effectiveness of Bet/EG for stabilizing volatile components of *Lavandula stoechas* was demonstrated by Vladic et al. [19]. Although there are no previous data on Bet/LA stabilization properties, which was the most effective in this study, the potential stabilizing effect may result from intermolecular bonds formation between DES constituents and dill extract components, contributing to the stabilization of the entire mixture. Betaine is a strong hydrogen bond acceptor, while ethylene glycol and levulinic acid can function as both hydrogen bond acceptors and donors. Furthermore, carvone and limonene were previously reported as constituents of DESs [33,34]. Other minor dill components, due to their chemical characteristics and functional groups, can also play the role of hydrogen bond acceptors and donors, further enhancing bond formation and stability within the mixture. For instance, components with functional groups (e.g., hydroxyl, carbonyl, or ether) can engage in hydrogen bonding, strengthening the DES matrix. Carvone, with its carbonyl group, may act as a hydrogen bond acceptor, while other components with -OH or NH groups can function as hydrogen bond donors, thereby increasing overall stability. These intermolecular interactions could create a stable environment with lower volatility, which is advantageous for preserving sensitive compounds in the mixture.

3.3. Microbiological stability during storage

During storage, temperature and light can induce numerous transformation reactions and the formation of non-terpene components, potentially impairing the organoleptic characteristics and microbiological stability of extracts, as well as reducing their bioactivity. In the dill-control extract, acetic acid was detected with an increasing trend after 3 months which could be consequence of microbial instability.

To assess the microbiological stability of dill-control and dill-DES extracts, the total number of microorganisms, molds and yeasts, and spores of anaerobic bacteria were monitored over a 6-month storage period at room temperature. These parameters were analyzed at the start of the experiment (0 months) and after 6 months of storage (Table 2). All dill-DES samples demonstrated microbiological stability during the 6-month storage period, with microbial counts remaining below 1 cfu/mL (not detected). This stability was maintained even in the dill-control sample, despite the significant alteration in its chemical profile.

3.4. Alterations of antibacterial properties during storage

Dill-control and dill-DES systems were evaluated for antimicrobial activity at the beginning of the study and after 6 months of storage at room temperature under light/dark cycles conditions (Table 3). The minimum inhibitory concentration (MIC) values were established against two Gram-negative bacteria, *E. coli* and *P. aeruginosa*, and two Gram-positive bacteria, *B. subtilis* and *S. aureus*. These values are expressed as mg of extract per mL (mg_{ext}/mL) (Table 3). To investigate the contribution of DESs to the antibacterial activity of dill-DES mixtures, the antibacterial effects of pure DESs were also assessed. For a more straightforward comparison between pure DESs and extracts, the

Table 2
Monitoring of number of microorganisms (aerobic microbial count), molds and yeasts, and spores of anaerobic bacteria (cfu/mL) in dill-control and dill extracts dispersed in deep eutectic systems (on start/0 month and after 6 monts).

Sample	Number of microorganisms (cfu/mL)		Molds and yeasts (cfu/mL)		Spores of anaerobic bacteria (cfu/mL)	
	Start	After 6 months	Start	After 6 months	Start	After 6 months
Dill-Control	< 1	< 1	< 1	< 1	< 1	< 1
Dill-Bet/LA	< 1	< 1	< 1	< 1	< 1	< 1
Dill-Bet/Gly	< 1	< 1	< 1	< 1	< 1	< 1
Dill-Bet/EG	< 1	< 1	< 1	< 1	< 1	< 1

*Bet/LA-betaine/levulinic acid; Bet/EG-betaine/ethylene glycol; Bet/Gly-betaine/glycerol.

Table 3
Antibacterial activity (minimal inhibitory concentration expressed as mg_{ext}/mL) of dill extracts (dill-control and dill extract dispersed in deep eutectic solvents) at the 0 month and after 6-month storage at room temperature and under light/dark cycles.

Sample	*Gram-positive bacteria*		*Gram-negative bacteria*	
	Bacillus subtilis	***Staphylococcus aureus***	***Escherichia colii***	***Pseudomonas aeruginosa***
Dill-control 0 m	0.781	1.563	0.391	0.391
Dill-control 6 m	0.391	0.391	0.195	0.391
Dill-Bet/LA 0 m	0.039	0.039	0.078	0.049
Dill-Bet/LA 6 m	0.049	0.024	0.049	0.049
Dill-Bet/Gly 0	1.563	3.125	0.781	0.781
Dill-Bet/Gly 6	6.250	12.500	6.250	6.250
Dill-Bet/EG 0	3.125	3.125	1.563	1.563
Dill-Bet/EG 6	0.313	0.625	0.313	0.156

*Bet/LA-betaine/levulinic acid; Bet/EG-betaine/ethylene glycol; Bet/Gly-betaine/glycerol. The number next to sample name indicates 0 month (start of the experiment) and after 6-months storage.

results for pure DESs were expressed as % v/v (Table 4).

At the start of the experiment (0 month), the MIC of the dill-control extract was 0.391 mg/mL against Gram-negative bacteria. Against Gram-positive bacteria, the activity was weaker, with an MIC of 0.781 mg/mL against *B. subtilis* and 1.563 mg/mL against *S. aureus*. Among the dill-DES extracts, dill-Bet/LA0 exhibited the most potent activity (0.039–0.078 mg/mL), followed by dill-Bet/Gly0 (0.781–3.125 mg/mL) and dill-Bet/EG0 (1.563–3.125 mg/mL). Dill-Bet/Gly0 and dill-Bet/EG0 showed stronger activity against Gram-negative bacteria, while dill-Bet/LA0 was more effective against Gram-positive bacteria.

Previous studies have reported the antibacterial activity of dill

Table 4
Antibacterial activity (minimal inhibitory concentration (MIC) expressed as % v/v) of pure deep eutectic systems and dill extracts dispersed in deep eutectic systems.

Sample	*Gram-positive bacteria*		*Gram-negative bacteria*	
	Bacillus subtilis	***Staphylococcus aureus***	***Escherichia colii***	***Pseudomonas aeruginosa***
Bet/Gly	25.000	50.000	12.500	12.500
Bet/Eg	12.500	25.000	12.500	6.250
Bet/LA	25.000	25.000	50.000	25.000
Dill-control 0 m	1.563	3.125	0.781	0.781
Dill-control 6 m	0.781	0.781	0.391	0.781
Dill-Bet/LA 0 m	0.078	0.078	0.156	0.098
Dill-Bet/LA 6 m	0.098	0.049	0.098	0.098
Dill-Bet/Gly 0	3.125	6.250	1.563	1.563
Dill-Bet/Gly 6	12.500	25.000	12.500	12.500
Dill-Bet/EG 0	6.250	6.250	3.125	3.125
Dill-Bet/EG 6	0.625	1.250	0.625	3.125

essential oil and extracts. Garcez et al. [35] found that dill extracts obtained by hydrodistillation showed activity against *E. coli* and *S. aureus* with MICs below 25 mg/mL. Extracts obtained by supercritical extraction had MICs of 40 mg/mL against *E. coli* and 25 mg/mL against *S. aureus*. Additionally, Ruangamnart et al. [27] reported that dill oil had MIC values averaging 10 mg/mL against several bacteria, including *S. aureus* and *E. coli*, which is a weaker activity compared to the extracts in our study. In the same study, the two most abundant components, limonene and carvone, showed a more significant activity that varied according to different microorganisms and strains in the range of 0.3125–10 mg/mL, while according to two stains of *P. aeruginosa* it was higher than 10 mg/mL [27]. Different extraction techniques and process conditions significantly influence the composition and antimicrobial activity of extracts. Variations in plant origin, such as soil, climate, and harvest timing, also affect the chemical profile of dill extracts. Additionally, differences in microbial strains and testing conditions may impact efficacy, which could explain the higher activity observed in supercritical CO_2 extracts in our study.

The precise mechanism of the antimicrobial activity of essential oils and their constituents is not fully defined. It is believed that they disrupt the integrity of bacterial cell membranes and increase their permeability, thereby interfering with nutrient transport. Due to their lipophilic nature, the components of essential oils can penetrate bacterial membranes, causing leakage of ions, RNA, proteins, and other intracellular components, ultimately leading to cell death. Additionally, these components may denature membrane proteins, impairing membrane functionality and disrupting metabolic pathways. These combined mechanisms are suggested to be responsible for their antimicrobial effectiveness [36].

Observing the antibacterial activity of pure DESs (Table 4), it is evident that the different activity of dill-DES systems was conditioned by their presence. Namely, Bet/Gly0 and Bet/EG0 had lower MICs against Gram-negative than Gram-positive bacteria (6.25–12.50 % v/v and 12.50–50.00 % v/v, respectively), while the opposite was found for Bet/LA (25.00–50.00 % v/v according to Gram-negative, and 25.00 % v/v according to Gram-positive bacteria).

Although the pure DES systems Bet/Gly and Bet/EG exhibited more pronounced antibacterial activity against Gram-negative bacteria compared to the Bet/LA system, the dispersion of dill extracts in these DESs did not enhance the antibacterial activity with regard to the dill-control extract. This indicates that at the start, the Bet/Gly and Bet/EG systems did not demonstrate additive or synergistic antibacterial effects with the dill extract. In contrast, the dispersion of dill extract in Bet/LA resulted in a substantial increase in antibacterial activity, which can be attributed to synergistic or additive interactions between the dill extract and the Bet/LA components. Specifically, the dill-Bet/LA0 extract showed 20–40 times higher activity against Gram-negative bacteria and 5–8 times stronger activity against Gram-positive bacteria compared to the dill-control extract. Therefore, the mutual interactions between the constituents of dill extracts and DES systems in the mixtures led to a decrease in antibacterial activity for dill-Bet/Gly0 and dill-Bet/EG0, but a significant increase in potency for the dill-Bet/LA0 system.

It has been reported that DES systems and their individual components do not exhibit the same activity, as the activity of the system does not represent a simple sum of the activities of its individual constituents. DES systems are mixtures with unique properties that can differ significantly from those of their individual components [37]. Therefore, it cannot be conclusively stated that the enhanced antibacterial effect of the dill-Bet/LA system is solely attributable to the presence of levulinic acid, but rather to the joint activity of dill components and Bet/LA system. However, levulinic acid itself has established antimicrobial potential. For example, the activity of levulinic acid has been evaluated both individually and in combination with the surfactant sodium dodecyl sulfate. While levulinic acid alone exhibited limited activity against *S. aureus*, its combination with sodium dodecyl sulfate

demonstrated significant synergistic bactericidal efficacy against *S. aureus*. This synergistic effect highlights its potential applications in food processing [38]. Furthermore, a combination of levulinic acid and *p*-anisic acid has been patented as an antiseptic product to enhance storage stability and prevent microbial contamination [39]. These findings emphasize the potential of levulinic acid in antimicrobial applications, particularly when used in synergistic formulations.

After 6 months of storage, the dill-Bet/LA sample exhibited the most consistent antimicrobial activity, reflecting the previously established greater chemical stability of this sample. In contrast, the other samples showed varying degrees of change in activity either an increase or decrease during storage, indicating a decline in their stability likely due to alterations in their chemical profiles over time. The significant decrease in the activity of dill-Bet/Gly correlates with a marked reduction in limonene, which has been reported to effectively inhibit *S. aureus* growth, likely by destabilizing the cell structure and causing leakage of nucleic acids and proteins [40]. Interestingly, after 6 months, dill-Bet/Gly showed an increase in carvone content to 80.35 %, a compound with documented yet limited antibacterial activity. Aggarwal et al. [41] reported the activity of carvone against various human pathogenic bacteria. However, according to the study of Kotan et al. [42], where the antibacterial activity of different pure monoterpenes was tested, weak antibacterial activity of carvone was established. The combined antibacterial activity of limonene and carvone has not been extensively studied, but a 3:2 ratio of these compounds exhibited effective repellent activity, indicating the importance of their ratio for achieving a synergistic effect [43]. Therefore, the increased carvone and decreased limonene in the dill-Bet/Gly extract likely contributed to the observed decline in antibacterial activity.

The dill-control sample showed a significant decrease in limonene during storage but an increase in antibacterial activity during 6-month storage. This could be attributed to a significant rise in α-phellandrene, a component known for its antimicrobial properties by increasing membrane permeability and fluidity, leading to cellular content leakage [44]. Essential oils rich in α-phellandrene have demonstrated activity against multiple bacteria [5], including *P. aeruginosa* and *S. aureus* [45].

The activity of the systems can be partially attributed to the dominant components of the extract; however, it is not solely determined by their presence. The overall activity results from the combination of all constituents, including minor ones, which can either enhance or reduce activity. Individual components may exhibit limited activity, but the presence of multiple terpene components can result in significant and enhanced antibacterial effects. The sensitivity of bacteria to essential oil and extracts and its primary components varies, suggesting that the activity arises from the synergistic interaction of all components, not just the dominant ones [46].

Nonpolar monoterpene hydrocarbons, such as limonene and *p*-cymene, can enhance the activity of other lipophilic components and drugs due to their hydrophobicity [47]. For instance, *p*-cymene, detected in dill-DES extracts after 3 and 6 months of storage, has been shown to exhibit synergistic effects with other components. Synergism between *p*-cymene and thymol, as well as between *p*-cymene and carvacrol, has been reported, where the presence of the hydrocarbon component facilitates penetration into the lipid membrane, thereby enhancing the activity of the other component [48]. Additionally, minor compounds, such as the degradation products limonene and carvone, can impact overall activity.

An important aspect of total activity is the ratio between components [43]. Yamaguchi et al. [49] tested the activity of individual terpenes and their combinations against various bacteria (*Salmonella enteritidis*, *E. coli*, *Acinetobacter baumannii*, *B. cereus*, *Enterococcus faecalis*, *S. aureus*, *Listeria monocytogenes*, and *Corynebacterium diphtheriae*). The combined activity of thymol and carvacrol was stronger compared to their individual activities, with lower concentrations of the mixture being more effective than higher concentrations of individual components. Similarly, combinations such as menthol/thymol and menthol/geraniol showed synergistic effects on the growth inhibition of *S. aureus* and *B. cereus*, whereas menthol alone did not exhibit antimicrobial activity [47].

3.5. Selection of correlated variables for exploratory data analysis

To better evaluate the relationships between constituents of dill extracts and their antibacterial activities against *B. subtilis*, *S. aureus*, *E. coli*, and *P. aeruginosa*, a correlation analysis was performed, resulting in a correlation matrix shown in Fig. 3.

The constituents of dill extracts exhibited varying degrees of correlation with antibacterial activities, classified as moderate (0.41–0.60), strong (0.61–0.80), and very strong (0.81–1.00) according to Evans [50]. These correlations, both positive and negative, were selected for further exploratory data analysis.

As shown in Fig. 3, strong positive correlations were observed for *p*-cymene (pCYM) and *cis*-dihydrocarvone (cDCRN) against *S. aureus* (0.73 and 0.61) and *E. coli* (0.73 and 0.64). Moderate positive correlations were noted for *p*-cymene (pCYM) and *cis*-dihydrocarvone (cDCRN) with activity against *B. subtilis* (0.45 and 0.46) and *P. aeruginosa* (0.59 and 0.52). Additionally, limonene (LIM) showed a moderate positive correlation with activity toward *B. subtilis* (0.40), and dill ether (DILE) exhibited a positive correlation with activity against *E. coli* strain (0.43).

Conversely, strong negative correlations were observed between *trans*-dihydrocarvone (tDCRN) and activity against all investigated microbial strains: *B. subtilis* (-0.79), *S. aureus* (-0.75), *E. coli* (-0.66), and P. *aeruginosa* (-0.79). α-pinene (αPIN) also showed strong negative correlations with activity toward *B. subtilis* (-0.62), *S. aureus* (-0.60), and *P. aeruginosa* (-0.64). Sabinene (SAB) exhibited a strong negative correlation with activity against *P. aeruginosa* (-0.61). Moderate negative correlations were found for carvone (CRN) against *B. subtilis* (-0.48) and *P. aeruginosa* (-0.44).

The key contributing compounds, *p*-cymene (pCYM), *cis*-dihydrocarvone (cDCRN), limonene (LIM), dill ether (DILE), trans-dihydrocarvone (tDCRN), α-pinene (αPIN), sabinene (SAB), and carvone (CRN), along with their antibacterial activity values, were selected and normalized for further exploratory data analysis via PCA approach. The resulting PCA bi-plot and loadings plot in two dimensions (Dim1 vs. Dim2) are presented in Fig. 4a and b.

In this process, 12 principal components were generated, corresponding to the number of variables in the input dataset. Each component explains a percentage of the total variance in the data, with the first principal component explaining about 55 % and the second one explaining 24.9 %. This indicates that more than half of the data variance can be represented solely by the first principal component. The cumulative proportion of Dim1 and Dim2 accounts for nearly 80 % of the total variance, implying that the first two principal components can accurately represent the input data.

The obtained PCA bi-plot, shown in Fig. 4a, illustrates that the investigated DES containing betaine and levulinic acid exhibit the highest antibacterial potentials towards all investigated bacterial species, both at the beginning (dill-Bet/LA 0) and after 6 months of observation (dill-Bet/LA 6). In contrast, the other systems displayed weaker activities and were grouped together at both the beginning (Cont 0, dill-Bet/Gly 0, and dill-Bet/EG 0) and after 6 months (Cont 6, dill-Bet/Gly 6, and dill-Bet/EG 6). The square cosine (cos2) loadings plot in Fig. 4b explains the representation of each variable in a given component. Variables grouped together are positively correlated, while negatively correlated variables are positioned on opposite sides. Low cos2 values, shown in brown and black, indicate poor representation by that component, whereas high cos2 values, shown in green and orange, indicate a good representation. High cos2 attributes, such as LIM and cDCRN (colored in green), have strong positive correlations. The cos2 attribute of pCYM (colored in orange) shows moderate positive correlations against all investigated bacterial strains, indicating the major role of these compounds in preserving the antibacterial potential of the

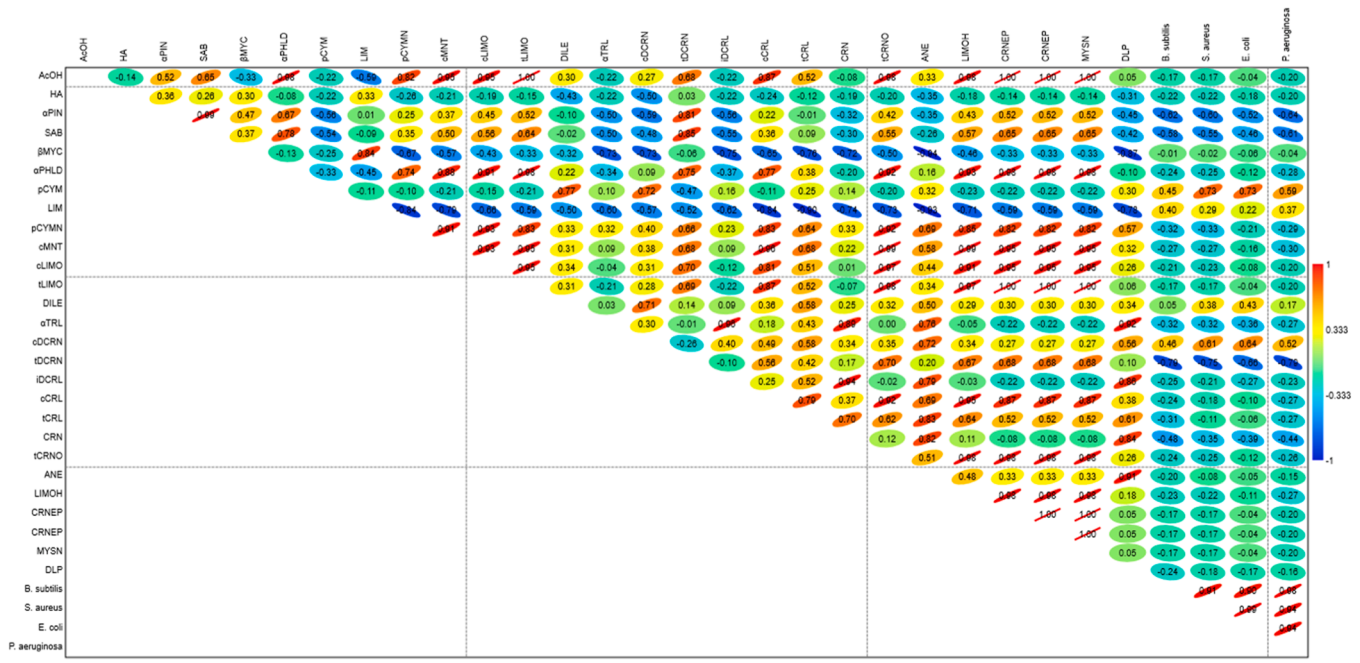

Fig. 3. Correlation matrix exhibiting relationships between quantified volatile compounds and achieved antibacterial activities against the strains of *Bacillus subtilis*, *Staphylococcus aureus*, *Escherichia coli*, and *Pseudomonas aeruginosa*.

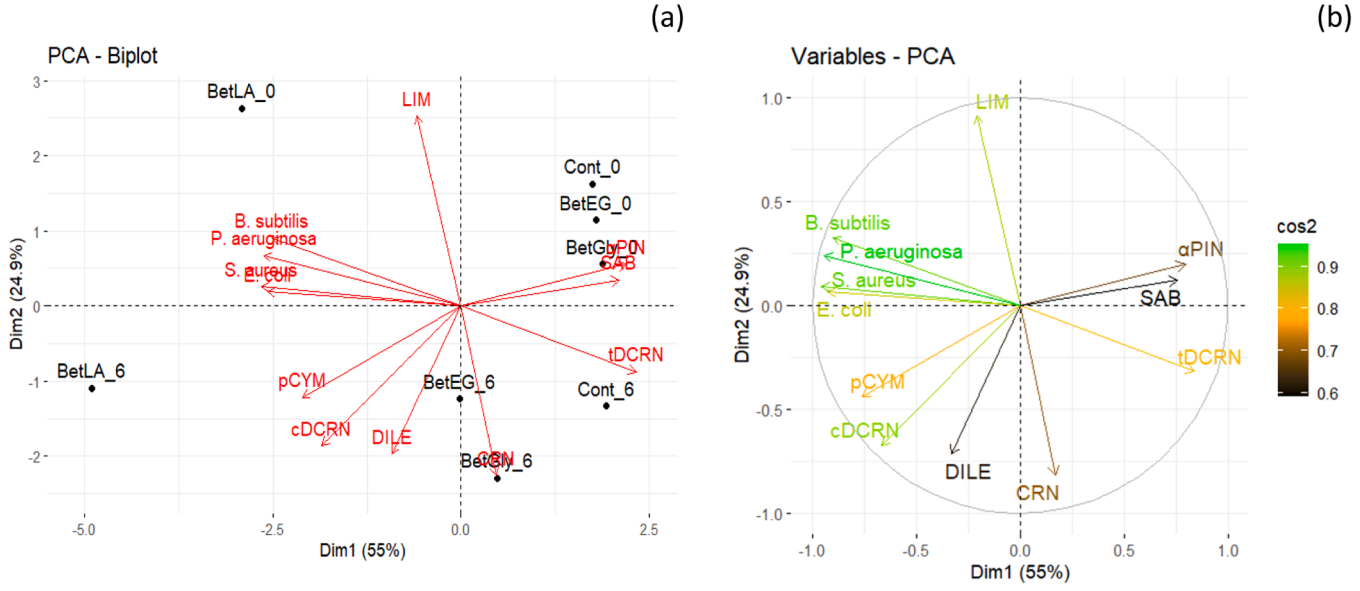

Fig. 4. Results of the principal component analysis in two PC dimensions (Dim1 vs. Dim2) showing: (a) bi-plot of investigated deep eutectic systems and selected volatiles with antibacterial potential; (b) square cosine loadings plot of selected volatiles impacting antibacterial potential.

investigated eutectic systems. Conversely, tDCRN has a moderate negative impact on preserving antibacterial potential, while CRN, αPIN, SAB, and DILE (shown in brown and black) have a lower influence on the antibacterial potential of the observed systems.

Establishing exact relationships is challenging due to rapid transformation reactions, but it is evident that both major and minor compounds impact the system's overall activity. These interactions can lead to increases or decreases in activity, as confirmed by the correlation matrix and principal component analysis. For instance, the negative impact of some minor compounds could be explained by their formation due to degradation of other components that are stronger antibacterial contributors. Additionally, during the transformation of components, the ratio of compounds changes, which is also a determining factor in the overall antibacterial response.

4. Conclusion

This study highlights the significant potential of supercritical CO_2 and DESs in enhancing the extraction, stabilization, and antimicrobial efficacy of volatile compounds from dill. By integrating supercritical CO_2 extraction with DESs, we have demonstrated a robust method for preserving both the chemical integrity and antimicrobial activity of dill extracts over a six-month storage period. The results indicate that the dill-Bet/LA system is particularly effective, showing significant improvements in antibacterial activity and stability compared to other systems and dill-control. This study provides a framework for advancing

sustainable production processes, aligning with the growing demand for environmentally friendly industrial solutions. This method could be particularly valuable in industries such as food, pharmaceuticals, and cosmetics, where stability is crucial. Supercritical CO_2 extraction reduces the need for harmful solvents, while DESs eliminate the need for refrigeration or synthetic preservatives, contributing to a more sustainable and efficient process. However, further testing is required to assess the toxicity and application-specific performance of DES-containing extracts. Additionally, life cycle and cost analyses are essential to evaluate the feasibility of large-scale applications, as scaling supercritical CO_2 extraction for industrial use presents challenges such as high capital costs and specialized equipment.

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CRediT authorship contribution statement

Ana Rita C. Duarte: Writing – review & editing, Supervision, Resources, Conceptualization. **Kristian Pastor:** Writing – original draft, Visualization, Software, Data curation, Conceptualization. **Krunoslav Aladic:** Validation, Investigation, Formal analysis, Data curation. **Stela Jokic:** Writing – review & editing, Validation, Supervision, Methodology, Conceptualization. **Jelena Vladic:** Writing – original draft, Resources, Project administration, Methodology, Investigation, Funding acquisition, Conceptualization. **Dragoljub Cvetkovic:** Writing – review & editing, Investigation, Formal analysis. **Valentina Pavic:** Writing – review & editing, Validation, Investigation, Formal analysis, Conceptualization. **Igor Jerkovic:** Writing – review & editing, Validation, Software, Resources, Conceptualization.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

No data was used for the research described in the article.

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