UNIVERSITÀ DEGLI STUDI DI PADOVA
Department of Comparative Biomedicine and Food Science

Second Cycle Degree (MSc) in Biotechnologies for Food Science

# ***Quantitative*** **Metabolomic Profiling of Terpenes and Fatty Acids in the Milk of Dairy Cows Supplemented with Officinal Plants Savory (*Satureja hortensis L.*) and Hemp (*Cannabis sativa L.*): An Analytical Investigation Utilizing GC×GC and GC-MS**

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**To my loving partner, family, and friends**

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# Abbreviations and Acronyms

| | |
|---|---|
| FA | Fatty Acid |
| SFA | Saturated Fatty Acid |
| MUFA | Monounsaturated Fatty Acid |
| PUFA | Polyunsaturated Fatty Acid |
| GCXGC | Two-Dimensional Gas Chromatography |
| HS-SPME – GC-MS | Headspace Solid-Phase Microextraction - Gas Chromatography-Mass Spectrometry |
| VOCs | Volatile Organic Compounds |
| pVOCs | Plant Volatile Organic Compounds |
| THC | Tetrahydrocannabinol |
| CBD | Cannabidiol |
| EU | European Union |
| CN Code | Customs Nomenclature Code |
| GPP | Gross Primary Productivity |
| SKE | Satureja khuzestanica |
| NSCLC | Non-Small Cell Lung Cancer |
| MVA | Mevalonic Acid |
| MEP | Methylerythritol Phosphate pathway. |
| PTR-MS | Proton-Transfer Reaction–Mass Spectrometry |
| ROS | Reactive Oxygen Species |
| DMAPP | Dimethylallyl Diphosphate. |
| PDO | Protected Designation of Origin |
| LCFAs | Long-Chain Fatty Acids |
| PGI | Protected Geographical Indication |
| HFL1 | Human Fetal Lung Fibroblast Cells |
| EPA | Eicosapentaenoic Acid |
| DHA | Docosahexaenoic Acid |
| CLA | Conjugated Linoleic Acid |
| HDL | High-Density Lipoproteins |
| LDL | Low-Density Lipoproteins. |
| CRP | C-reactive Protein |
| IL | Interleukin |
| CHD | Coronary Heart Disease |
| SREBP | Sterol Regulatory Element-Binding Proteins |
| HSO | Hemp Seed Oil |
| ARAV | Associazione Regionale Allevatori Veneto |
| D.M | Dry Matter |
| PG | crude protein |
| EE | ethereal extract |
| NDF | neutral detergent fiber |
| FAME | fatty acid methyl esters |
| TAGs | Triglycerides |
| mTOR | Mechanistic Target of Rapamycin |
| MEP | 2-C-methyl-D-erythritol-4-phosphate pathway |
| SCFAs | Short-Chain Fatty Acids |

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# Abstract

This thesis delves into a comprehensive exploration of the impact of dietary supplementation with hemp (*Cannabis sativa* L.) and savory (*Satureja hortensis* L.) on the composition of volatile organic compounds and fatty acids in the milk of dairy cows. The study aimed at elucidating the intricate interplay between dietary supplementation with hemp and savory and the resulting aromatic and nutritional characteristics of dairy milk. By focusing on the identification and quantification of terpenes and fatty acids, the research resulted in providing valuable insights into how these dietary additives influence milk composition. The experimental 3×3 Latin square design employed in this study involved controlled dietary treatments where dairy cows were supplemented with hemp and savory, while a standard diet served as the control group. This design allowed for a systematic comparison of milk composition across different dietary regimens, enabling the assessment of the specific effects of hemp and savory supplementation. Milk samples collected throughout the study period underwent rigorous analysis using Two-Dimensional Gas Chromatography (GC×GC) Headspace Solid-Phase Microextraction Gas Chromatography-Mass Spectrometry (HS-SPME GC-MS) to identify and quantify terpenes and fatty acids. The comprehensive nature of the analysis revealed the presence of 29 terpenes and 87 fatty acids in the milk samples, providing a detailed snapshot of milk composition under various dietary conditions. One of the key findings of the study was the enrichment of milk with terpenes following supplementation with hemp and savory. This observation underscores the potential of these dietary additives to modulate the aromatic profile of dairy milk, potentially enhancing its sensory properties and value-added attributes. Furthermore, the study highlighted the individual variability in aromatic and fatty acid profiles among dairy cows, emphasizing the complex interplay between feeding behaviour, digestive processes, and metabolic transfer mechanisms. While hemp and savory supplementation demonstrated notable effects on milk terpene content, the impact on the milk's fatty acid profile was comparatively limited, suggesting a need for further investigation into the underlying mechanisms. In conclusion, this research contributes significant insights into the nuanced interactions between dietary components, individual variation, and milk composition in dairy cows supplemented with hemp and savory. By unravelling the complex dynamics governing milk composition, the study paves the way for informed decision-making in animal nutrition and dairy production practices, ultimately enhancing the quality and nutritional value of dairy products.

# Chapter 1

# Introduction

In the domain of livestock nutrition and dairy science, the intricate relationship between dietary components and the composition of dairy products stands as a focal point of research. An intriguing aspect within this field involves the scrutiny of fatty acid (FA) profiles in dairy products, notably milk. These FAs play a pivotal role not only in determining the nutritional quality of dairy products but also in impacting human health (Hanus et al., 2018). Consequently, understanding the factors influencing these FA profiles is of paramount importance. The dietary regimen of dairy cows, which includes various feed components, has been a subject of extensive research. Researchers have explored the potential of dietary interventions to influence the FA composition of milk, aiming to enhance their nutritional value and meet consumer demands for healthier food options. Moreover, the inclusion of aromatic plants in the diet of dairy cows has emerged as a particularly intriguing avenue within this context (Caroprese et al., 2019). Beyond fatty acids, another compelling dimension of research in this field pertains to the analysis of volatile organic compounds (VOCs) present in dairy products. These VOCs contribute to the aroma and flavour of milk, making them a captivating area of investigation to meet the sensory demands of consumers seeking unique flavour experiences (Yuan et al., 2023a). As such, understanding the intricate interplay between dietary factors, FA profiles, and VOCs is essential for advancing the quality and desirability of dairy products. This research investigates the complex interplay between bovine dietary composition, particularly the inclusion of the aromatic plants Hemp *(Cannabis sativa* L.*)* and Savory *(Satureja hortensis* L.*)*, and their effects on the composition of milk. The analysis goes beyond fatty acid profiles to encompass the presence of VOCs, which significantly influence the sensory attributes of this dairy product.

## 1.1 Hemp *(Cannabis Sativa L.)*

Cannabis sativa L., commonly referred to as hemp, is an herbaceous, plant from the Cannabaceae family. It is one of the oldest growing plants known to man, due to its long cultivation history, and it can't be identified with certainty where their origins come from (Farinon et al., 2020). Due to its capacity for production of food, textiles, clothing, organic plastic, paper, paint, biofuels and animal feed as well as illumination oil, hemp is one of the most valuable crops in the world with great societal and economic value (Cerino et al., 2021). Hemp seeds have earned the designation of "superfoods" owing to their exceptional omega-6 to omega-3 fatty acid ratio (3:1) suitable for human dietary needs and their distinctive amino acid composition, rendering them a highly valuable food source. Hemp, along with hemp oil

and hemp seed meal, offers a plentiful supply of proteins, polyunsaturated fats, as well as vitamins and minerals, making them a versatile ingredient for various plant-based food products (Szumny & Żołnierczyk, 2023).

### 1.1.1 Botanical Characteristics of Hemp

*Figure 1. Cannabis Sativa, Hemp seeds (Wikipedia).*

*Table 1.Classification of Hemp (https://plants.usda.gov/home/classification/70749).*

| Rank | Scientific Name and Common Name |
|---|---|
| **Kingdom** | *Plantae* -Plants |
| **Subkingdom** | *Tracharpionta* - Vascular plants |
| **Superdivision** | *Spermatophyta* - Seed plants |
| **Division** | *Magnoliophyta* - Flowering plants |
| **Class** | *Magnoliopsida* - Dicotyledons |
| **Subclass** | *Hamamelididas* |
| **Order** | *Urticales* |
| **Family** | *Cannabacea Martinov* - Hemp family |
| **Genus** | *Cannabis L.* - hemp |
| **Species** | *Cannabis sativa L.* - marijuana |

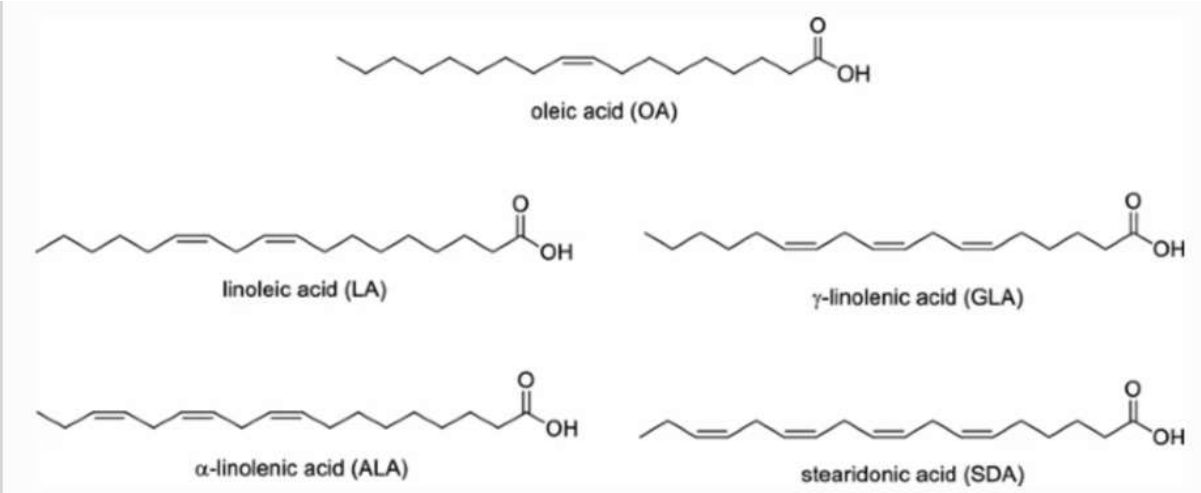
The taxonomic classification of *Cannabis sativa* L. has been a subject of debate for numerous years. Presently, it is established that hemp belongs to the order Rosales, family Cannabaceae, genus Cannabis, and the species is denoted as *C. sativa* L. This species encompasses both industrial hemp (*C. sativa* L. var. sativa) and the psychoactive variety, *C. sativa* L. var. indica. Within the realm of hemp, distinct genetic groups emerge, each exhibiting adaptability to

varying geographical and climatic conditions, along with the capacity to thrive in diverse substrates. The categorization of hemp groups can be based on several criteria, with the most significant ones being their origin, length of vegetative growth, and cannabinoid content (THC and CBD) (Strzelczyk et al., 2022). The primary distinguishing factor when considering the various intended uses of *C. sativa* L. is the concentration of its two predominant and well-known phytochemicals. These compounds are pivotal in defining the plant's purpose. The first is tetrahydrocannabinol THC, the sole psychoactive and toxic compound in the plant, while the second is cannabidiol (CBD), which lacks psychoactive effects. Both THC and CBD fall under the category of cannabinoids, which consists of over 100 secondary metabolites within the terpenophenolics compound family, a characteristic shared by all *C. sativa* L. plants (Farinon et al., 2020). Hemp, scientifically known as *Cannabis sativa* L., belongs to the Cannabaceae family and is characterized by its exceptionally low tetrahydrocannabinol (THC) content (European Commission on Agriculture and Rural Development). (https://agriculture.ec.europa.eu/farming/crop-productions-and-plant-based-products/hemp_en). Being a bast fibre plant akin to flax, kenaf, and jute, it exhibits a distinctive structural composition. Its stem comprises a central hollow core, enclosed by a woody fibre layer known as "hurds". Beyond the cambium layer, responsible for cell growth and differentiation, lies the phloem or parenchyma layer housing the elongated cells recognized as bast fibres. Hemp plants exhibit imperfect flowers. In their natural state, hemp is dioecious, meaning there are distinct male and female plants. However, certain commercial varieties may be monoecious, indicating that a single plant can bear both male and female flowers. Male plants play a crucial role in the production of grains or seeds, as pollination is necessary to facilitate seed formation (X. Chen et al., 2021). The hemp plant's life cycle spans four to six months and includes four primary growth stages: germination and emergence, vegetative growth, flowering and seed formation, and senescence (Zampori et al., 2013). Hemp seeds are characterized by their smooth texture and measure between one-eighth to one-fourth of an inch in length. Typically, these seeds contain an oil content ranging from 29 to 34 %. This oil closely resembles drying oils like linseed and tung oil and is predominantly composed of three primary fatty acids: linoleic (54-60 %), linolenic (15-20 %), and oleic (11-13 %) (USDA) (https://www.ers.usda.gov/webdocs/publications/41740/15854_ages001ed_1_.pdf?v=0#:~:text=Hemp%20is%20a%20bast%20fiber,cells%20known%20as%20bast%20fiber). The growth requirements for hemp can vary depending on factors such as the specific variety of hemp, climate, and intended use. Hemp thrives in temperate climates with well-defined seasons. It prefers daytime temperatures between 60-80°F (15-27°C) during the growing season

(University of Missouri, 2019). In addition to that, a well-drained soil with a pH between 6.0-7.5 is ideal for hemp cultivation (Visković et al., 2023). Hemp has moderate water requirements but needs consistent moisture during the growing season. Drip irrigation or soaker hoses are often used to ensure even watering (University of Wisconsin-Madison, n.d.).

### 1.1.2 Chemical Composition of Hemp and Nutritional Significance

For thousands of years, hemp seeds from the Cannabis sativa L. plant have served as a significant nutritional source in Chinese and European cultures. However, in contemporary times, despite a wealth of clinical evidence emphasizing their substantial health-promoting properties, many individuals remain uninformed about the nutritional and nutraceutical advantages these seeds offer (Crescente et al., 2018). A recent proteomic analysis of hempseed has brought to light the fact that hempseed is an underutilized, non-legume seed that is rich in protein and possesses favorable nutritional characteristics. Hempseed proteins are renowned for their high digestibility and essential amino acid composition (Aiello et al., 2016) Among the amino acids found in hempseeds, arginine is present in notably high and unexpected quantities. This crucial metabolite serves as a metabolic precursor for the synthesis of nitric oxide (NO), which plays a crucial role as a signalling messenger within the cardiovascular system. Arginine is involved in controlling various cardiovascular functions, including hemostasis, fibrinolysis, interactions of platelets and leukocytes with the arterial wall, the regulation of vascular tone, the proliferation of vascular smooth muscle cells, and the maintenance of blood pressure homeostasis (Rodriguez-Leyva & Pierce, 2010). The carbohydrate content, which is estimated to be over 25% of the seed, significantly decreases to approximately 10% once the hard outer shell is removed. While various types of monosaccharides, disaccharides, and cyclitols have been identified as sugar components in Cannabis species, there is a lack of specific literature data that focus on them as a subject of interest (Brenneisen, 2007). Hemp seeds typically contain around 33–35% oil (Callaway, 2004a). The primary importance of hempseed and its oil lies in their fatty acid composition. This composition includes essential components from both the ω-3 and ω-6 classes (as illustrated in Fig. 3). These essential fatty acids play a vital role in numerous physiological processes, such as maintaining the structure of cell membranes, synthesizing prostaglandins and leukotrienes, and ensuring skin integrity (Crescente et al., 2018).




Main unsaturated fatty acids in hempseed and its oil. OA, LA and ALA constitute approximately the 90% of total fatty acid content

*Figure 2. Unsaturated Fatty Acids in Hempseed.*

Tocopherols, which are minor constituents of hempseed oily fraction, are well-known for their antioxidant properties, which help to maintain the oxidative stability of oils. As potent chain-breakers, these substances—which collectively make up vitamin E's various vitamers—can actually slow down and even reverse lipo-peroxidative radical chain reactions, halting the oxidation of PUFA-rich oils. (T. Chen et al., 2010).

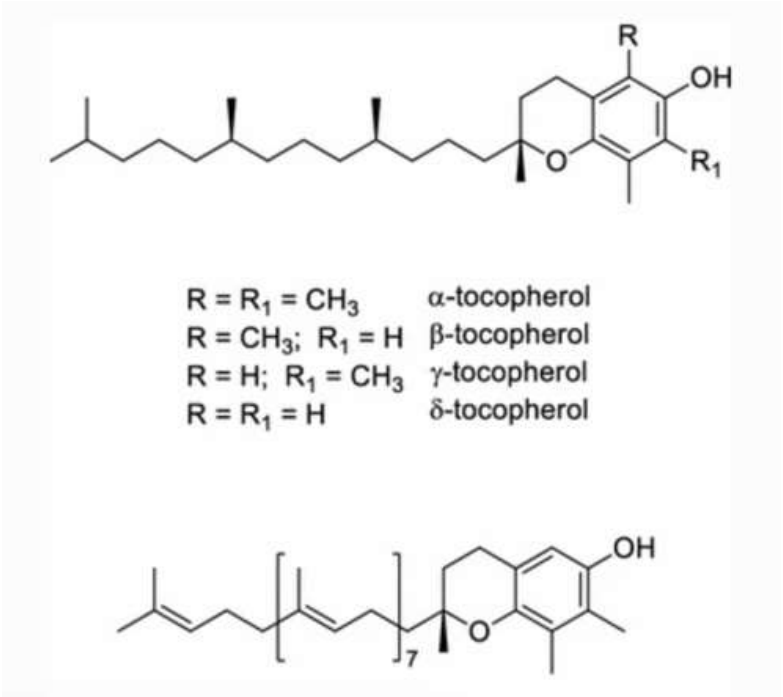



*Figure 3. Tocopherols and Their Analogues in Hemp Seed.*

The liposoluble components of hemp seed that are most important to human health are phytocannabinoids, which are a class of terpenophenolic compounds that are mostly found in Cannabis and are designated as C21 or C22 (for the carboxylated forms). The main 2 cannabinoids are tetrahydrocannabinol (THC) which is the principal psychoactive constituent of cannabis, and cannabidiol (CBD) which has a medical role.

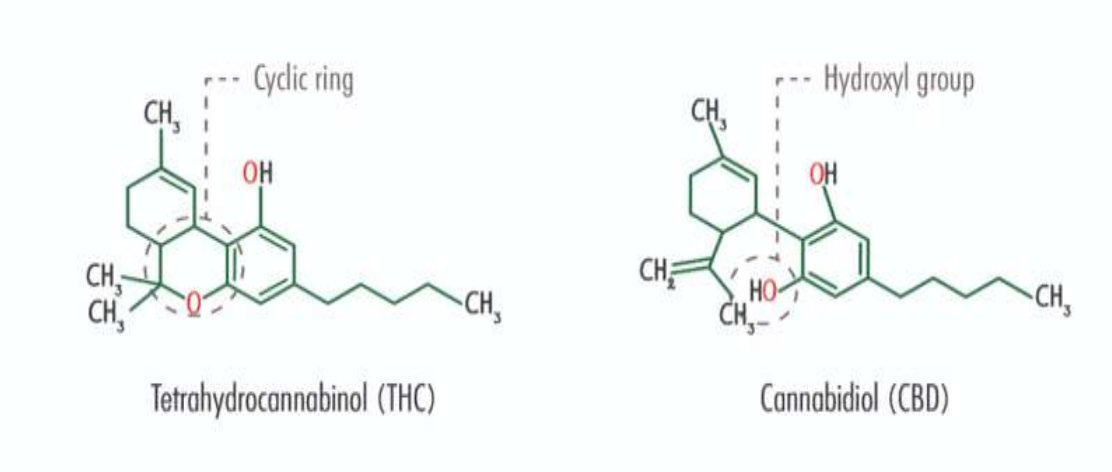



*Figure 4. Structural Formulas of THC and CBD (https://www.analyticalcannabis.com/articles/cbd-vs-thc-what-are-the-main-differences-297486).*

The typical terpene contents of hemp, depending on the variety, range from 0.25% to 0.278 percent weight in leaves and 1.283 percent to 2.14% in inflorescence on a dry basis. Terpenes are hydrocarbons that are chemically made up of tiny isoprene units, or five-carbon building blocks, that are connected to one another to form chains.It's interesting to note that the 10-carbon GPP molecule serves as a precursor for both terpenes and cannabinoids. Terpene and cannabinoid concentrations in hemp are therefore typically positively correlated (Hanuš & Hod, 2020). Cannabis's distinctive aroma is attributed to terpenes, which are the main components of essential oils. Terpenes exhibit entourage and/or synergistic effects with cannabinoids, and their interactions have only been the subject of conjecture in recent decades. Numerous terpenes have been found that reference the sensory qualities of cannabis and significantly influence consumer experiences and market value. They also strengthen the effects of many therapies, particularly aromatherapy (Sommano et al., 2020).

### 1.1.3 Hemp Agricultural Benefits

According to the European Commission on Agriculture and Rural Development, on the level of carbon storage, despite taking only five months to grow, one hectare of hemp can sequester

9 to 15 tonnes of $CO_2$, which is comparable to the amount sequestered by a young forest. Moreover, when hemp is utilized in crop rotation, it contributes to the disruption of disease cycles. Furthermore, the quick growth and ability of hemp plants to shade one another prevents weeds from sprouting. Hemp's dense leaves act as a natural soil cover to prevent soil erosion and water loss. After just three weeks of germination, hemp covers the entire ground. A lack of pollen production from other crops typically coincides with the flowering cycle, which takes place between July and September. A lot of pollen is produced by hemp. Additionally, hemp seeds are a food source for animals and a place for birds to live. Also, according to the European Commission on Agriculture and Rural Development, it requires or no use of pesticides since hemp lacks natural predators then it is less likely to be harmed by pests, so most of the time it is possible to avoid using insecticides, herbicides, and fungicides.

### 1.1.4 Hemp in Animal feed

In recent years, hemp seeds, hemp seed cakes, and hemp seed oil have been added to animal feed. Hempseed and hempseed cake can be a good source of protein and fat in the diet, while hemp oil is added to the feed to provide essential fatty acids (Della Rocca & Di Salvo, 2020). Hemp seeds, which come from the *Cannabis sativa* plant, are becoming more and more well-liked as a possible feed option for animals because of their many uses in animal husbandry and nutritional advantages (Skřivan et al., 2020). They are very nutrient-dense and contain vital nutrients that are good for a variety of livestock species. The high protein content of hemp seeds promotes growth in young animals and may aid in livestock weight gain. Every part of the hemp plant has a different nutritional value and can be used to make animal feed. The following are the main hemp plant parts used in animal feed: hemp seeds; because hemp seeds have a high protein content, which helps young animals grow and gain weight, they are especially sought after. Especially, they are abundant in vital nutrients and amino acids, which support healthy development and general animal well-being (Callaway, 2004), hemp seed meal, which is an important addition to animal diets that is produced as a byproduct of extracting oil from hemp seeds. It acts as a concentrated source of protein, increasing the amount of protein in animal feed and promoting the growth of healthy muscles (Gakhar et al., 2012). While hemp leaves and stalks are less frequently used in animal feed, they are still worth considering. These sections give livestock a balanced diet by providing extra nutrients and vital dietary fibre. They are particularly important for minimizing waste and optimizing the hemp plant's nutritional content (Gakhar et al., 2012). The high concentration of essential FA, especially Ω-3 and Ω-6, found in hemp seed oil is well known. The total FA composition of animal diets is improved

by adding hemp seed oil to animal feed. It is anticipated that this improvement will result in healthier animals and higher levels of output (Della Rocca & Di Salvo, 2020).

### 1.1.5 Hemp in Human Feed

Hemp hearts, often called hemp seeds, are gaining increasing popularity as a nutritious dietary addition. Their exceptional nutritional content and ability to be used in a wide range of culinary applications have made them highly sought after (Callaway, 2004). Hemp can be incorporated into various foods to enhance their nutritional value and add a unique flavour. Some common foods and products that can include hemp include snacks, smoothies, baked goods, yogurt, cereal, dressing, and edible oils. Hemp seeds are a rich source of essential nutrients, making them a valuable component of a balanced diet. Here are some key nutritional values per 3 tablespoons (30 grams) of hulled hemp seeds:

- Calories: Approximately 166 kcal
- Protein: About 10 grams
- Fiber: Approximately 1.2 grams
- Fats: Roughly 14 grams, mainly consisting of healthy fats, including Ω-3 and Ω-6 FAs
- Carbohydrates: About 3 grams
- Vitamins and Minerals: Hemp seeds provide essential vitamins and minerals such as vitamin E, potassium, magnesium, and iron (House et al., 2010).

Hemp seeds are renowned for their protein content, offering a full array of necessary amino acids. This outstanding protein composition renders them a valuable resource for vegetarians and vegans. Importantly, the protein found in hemp seeds is easily digested and has been assessed as a high-quality source of nutrition for humans (House et al., 2010). Hemp seeds are known for having an Ω-6 to Ω-3 ratio within the desirable range of 2:1 to 3:1. This balanced ratio is considered favourable for promoting overall health (Callaway, 2004a). The inclusion of these necessary fats not only promotes heart health but also assists in reducing inflammation in the body (Schwab et al., 2006). Different analysis showed various polysaccharides that were identified in different parts of the hemp seed, with the hulls showing variations in cellulose content ranging from 22.0% to 36.7% and xylan content ranging from 5.7% to 17.1% (Schultz et al., 2020). These fibres contribute to regular bowel movements and help with weight management by promoting a feeling of fullness and reducing overeating.

### 1.1.6 Cultivation of Hemp in Europe and Import Regulations

Hemp cultivation is widespread across Europe, and in recent years, the EU has seen a substantial expansion in the acreage dedicated to hemp farming. From 2015 to 2022, the area

under hemp cultivation increased by 60%, growing from 20,540 hectares to 33,020 hectares. During this same period, hemp production also saw a significant 84.3% increase, rising from 97,130 tonnes to 179,020 tonnes. Among EU member states, as shown in Figure 5, France stands as the largest hemp producer, contributing to over 60% of the total EU hemp production. Following France, Germany accounts for approximately 17% of EU hemp production, with The Netherlands contributing around 5%. Hemp is grown primarily for its industrial uses and there are 75 different hemp varieties registered in the EU catalogue, of which Asso and Carma, Carmaleonte, Codimono and Eletta Campana in Italy, out of the 54 cultivars that are shown in the Table 2. In accordance with Article 189 of Regulation (EU) 1308/2013, all imports of hemp are currently subject to an import license requirement. Additionally, specific regulations pertain to different hemp products:

1. Raw true hemp falling within CN code 5302 10 must have a THC content not exceeding 0.3%. This restriction is in place to ensure that imported hemp remains within the legal limit for THC content.
2. Hemp seeds intended for sowing purposes must be accompanied by proof that the THC content of the variety in question does not exceed 0.3%. This requirement aims to maintain the integrity of hemp strains grown for agricultural and industrial purposes.
3. Hemp seeds not designated for sowing may be imported only under the authorization of EU member countries, and authorized importers must provide evidence that the seeds have been processed in a way that excludes their use for planting. This provision ensures that non-sowing hemp seeds are not utilized for agricultural purposes.

These regulations are in place to manage the importation and use of hemp products within the EU, addressing various aspects of hemp production and trade to uphold legal and quality standards. (https://agriculture.ec.europa.eu/farming/crop-productions-and-plant-based-products/hemp_en).

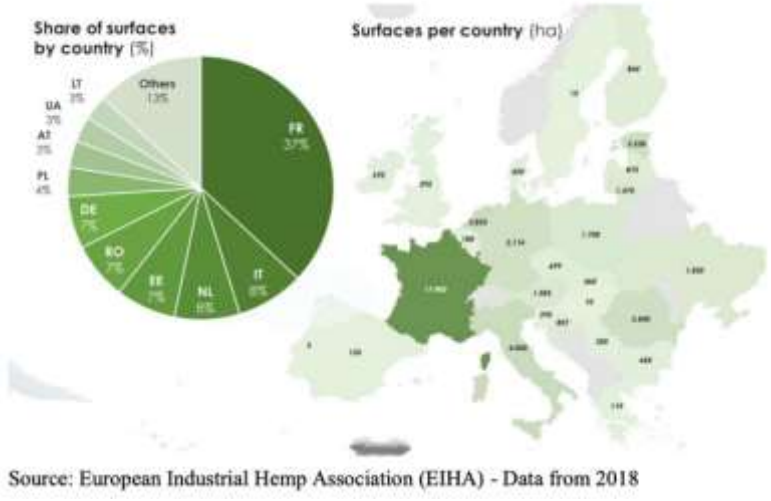



*Figure 5. Surfaces for hemp cultivation by Member States*

*Table 2. Industrial hemp cultivars registered and listed in the European Union Plant Variety Database and their origin.*

| No | Registered varieties | Origin | No | Registered varieties | Origin |
|---|---|---|---|---|---|
| **1** | Adzelviesi | Latvia | 33 | Ivory | Nederland |
| **2** | Armanca | Hungary | 34 | KC Bonusz | Hungary |
| **3** | Asso | Switzerland | 35 | KC Dora | Hungary |
| **4** | Austa SK | Czech Republic | 36 | KC Virtus | Hungary |
| **5** | Balaton | Nederland | 37 | KC Zuzaba | Hungary |
| **6** | Beniko | Poland | 38 | KCA Borana | Hungary |
| **7** | Bialobrzeskie | Czech Republic | 39 | Kompoliti | Hungary |
| **8** | Cannakomp | Polan | 40 | Kompoliti Hibrid TC | Hungary |
| **9** | Carma | Hungary | 41 | Lipko | Hungary |
| **10** | Carmaleonte | Italy | 42 | Lovrin 110 | Roamnia |
| **11** | Chameleon | Italy | 43 | Marcello | Nederland |
| **12** | Codimono | Nederland | 44 | Markant | Nederland |
| **13** | Dacia Secuieni | Italy | 45 | Monoica | Switzerland |
| **14** | Delta-405 | Romania | 46 | Orion 33 | Hungary |
| **15** | Delta-llosa | Spanish | 47 | Rajan | France |
| **16** | Dioica 88 | Spanish | 48 | Ratza | Poland |
| **17** | Earlina 8 FC | France | 49 | Santhica 23 | Romania |
| **18** | Eletta Campana | France | 50 | Santhica 27 | France |
| **19** | Epsilon 68 | Italy | 51 | Santhica 70 | France |
| **20** | Fedora 17 | France Switzerland | 52 | Secuieni Jubileu | France |

## 1.2 Savory *(Satureja hortensis L.)*

Species within the genus Satureja L., commonly known as savory, are members of the Lamiaceae family. This genus encompasses approximately 200 species of herbs and shrubs, primarily cultivated in regions such as the Mediterranean, Europe, West Asia, North Africa, the Canary Islands, and South America (Momtaz & Abdollahi, 2010).Summer savory (*Satureja hortensis* L.) stands out as one of the favoured types of savory, known for its seasonal nature. This herb, which shares comparable functions and flavours with the perennial winter savory, sees more frequent usage. Autumn savory is preferred for its bitter taste. Blossoming with violet tubular flowers from July to September in the Northern Hemisphere, this herb features relatively slender brass foliage and reaches a height ranging from 30 to 60 cm (1 to 2 ft). *Satureja hortensis* L. holds a notable status as a herb in eastern Canada and can be utilized in a manner akin to sage (Burlando et al., 2010). Satureja species have attracted growing attention among medicinal plants due to their composition of various bioactive chemicals. These include volatile oils, phenolic compounds, flavonoids, tannins, steroids, acids, gums, mucilage, pyrocatechols, and other constituents (Momtaz & Abdollahi, 2010) Research has demonstrated a wealth of diversity in the chemical composition and therapeutic properties across various Satureja species. The primary constituents found in the essential oils of savory species include carvacrol, thymol, phenols, and flavonoids. Numerous studies highlight that, in *S. hortensis*, the major components of the essential oil are carvacrol, thymol, γ-terpinene, and ρ-cymene, while smaller amounts of α-terpinene, β-caryophyllene, and β-bisabolene are also present (Gursoy et al., 2009). The elevated levels of polyphenols and flavonoids, coupled with the ease of cultivation and appealing fragrance, contribute to the utilization of savory species in food preparation. Additionally, these qualities make savory a desirable ingredient in the cosmetic and pharmaceutical industries (Momtaz & Abdollahi, 2010). Dried summer savory typically contains around 1% of volatile oil, with carvacrol and thymol as the primary constituents, along with monoterpene hydrocarbons like β-pinene, ρ -cymene, limonene, and camphene. The leaves also contain various minor components, including minerals and vitamins. Winter savory contains approximately 1.6% of volatile oil. Different sources may document varying dominant components of the oil, with some indicating caryophyllene and geraniol, while others point to carvacrol (Hamidpour et al., 2014). Savory species have a history of traditional medicinal use for addressing various ailments and their symptoms. They have been employed to alleviate muscle pain, as well as to address stomach and intestinal disorders, including conditions like nausea, indigestion, and diarrhoea. Additionally, savory has been used in traditional medicine to address infectious diseases (Momtaz & Abdollahi, 2010).

### 1.2.1 Botanical Characteristics of Savory

*Table 3. Taxonomy of Satureja Hortensis L.*

| | |
|---|---|
| **Domain** | **Eukaryota** |
| **Kingdom** | *Plantae* |
| **Subkingdom** | *Tracheobionta* |
| **Superdivision** | *Spermatophyta* |
| **Division** | *Magnoliopsida* |
| **Subclass** | *Asteridae* |
| **Order** | *Lamiales* |
| **Family** | *Lamiaceae* |
| **Type** | *Satureja* |

Savory comprises the dried leaves and flowering tops of *Satureja hortensis L.* (Lamiaceae). The leaves are characterized by their green colour, nearly sessile nature, entire margins, and a linear-oblong to spathulate shape when unfolded (Bagchi & Srivastava, 2003).Glandular punctate, these leaves are acute to rounded at the apex, tapering at the base, measuring 12–35 × 1–2 mm, and exhibit a pubescent texture. The flowers are tubular-bilabiate, purple, and can reach up to 5 mm in length in leaf axils. The calyx is campanulate, hispid on nerves, and has five subulate teeth. The corolla measures about 4–5 mm in length. Stamens are four and didynamous, and the style features a bilobed stigma (Bagchi & Srivastava, 2003). In the surface view, the epidermal cell walls on both the upper and lower surfaces exhibit sinuate and beaded characteristics, while the stomata appear elliptical. The non-glandular hairs are abundant, uniseriate, and consist of up to six cells, terminating in a pointed distal cell. The glandular hairs are also numerous, featuring a head composed of up to 12 cells and a one-celled stalk (Bagchi & Srivastava, 2003) Savory is characterized by a distinctly aromatic odour, and its taste is both aromatic and warm. The leaves are typically gathered when the plants are in flower, and they undergo a drying process in the shade or indoors with the circulation of warm air before being packaged (Wilson, 2016).

*Figure 6. Satureja Hortensis L. (https://www.magicgardenseeds.com/The-Aromatic/Summer-Savory-%28Satureja-hortensis%29-A.1004-).*

### 1.2.2 Cultivation of Savory

The cultivation, pollination, and seed propagation of *Satureja hortensis*, commonly known as summer savory, represent crucial aspects of its agricultural management. Cultivation practices involve considerations such as soil type, sunlight exposure, and nutrient requirements. Optimal growth conditions typically include well-drained soils, ample sunlight, and the application of essential nutrients, with phosphorus oxide (40-60 kg/ha) and potassium oxide (40-50 kg/ha) being key components (Lis-Balchin, 2006). The primary mode of pollination in *Satureja hortensis* is entomophily, involving insects such as bees and ants, which significantly contributes to the plant's reproductive success. The seeds, enclosed in trigonal ovoid nucleus, naturally fall to the ground and undergo dispersal, with each seed weighing approximately 45 to 50 mg. The process of seed propagation is supported by natural dispersal mechanisms, eliminating the need for intricate cultivation details. A frequent practice for ensuring viable seeds for subsequent plantings is seed curing. These combined factors enhance the resilience and adaptability of *Satureja hortensis*, making it well-suited for both culinary and medicinal applications.

### 1.2.3 Chemical Composition of Savory and Nutritional Significance

The raw material of *Satureja hortensis* L. has been analysed for its composition, revealing significant amounts of minerals. These include potassium (ranging from 1.68 to 3.38 $mg \cdot kg^{-1}$ dry matter), phosphorus (0.31–0.72 $mg \cdot kg^{-1}$ dry matter), calcium (1.08–2.84 $mg \cdot kg^{-1}$ dry matter), magnesium (0.25–0.61 $mg \cdot kg^{-1}$ dry matter), iron (242–726 $mg \cdot kg^{-1}$ dry matter), and sodium (0.007–0.013 $mg \cdot kg^{-1}$ dry matter) (Mumivand et al., 2010). The mineral composition of plants is crucial not only for the nutritional content of food but also for the development, growth, and yield of crops. Essential oils derived from various species within this family have essential functions. These oils play a role in both physiological functions, such as photosynthesis, and ecological functions, involving the interactions between the flowers and their environment. Additionally, there is notable

variation in the chemical structure of oils obtained from different strains of Satureja (Slavkovska et al., 2001). Several studies have demonstrated that the primary compounds found in Satureja species include tannins, volatile oils, sterols, acids, gums, pyrocatechol, phenolic compounds, and mucilage. The content of the oils is influenced by climatic, seasonal, and soil conditions, along with the harvest period and extraction methods (Baydar et al., 2004). For chemical profiling, essential oils from air-dried plants and robust spores were extracted using water through a 4-hour distillation process in a Clevenger-type apparatus. The leaves and seeds of summer savory were found to contain a total of 23 and 24 components, respectively (Farmanpour-Kalalagh et al., 2020). Additionally, summer savory seeds contain chemicals such as Carvacrol, Estragole (Methyl Chavicol), Caryophyllene, and E-Caryophyllene, while the leaves are rich in Carvacrol, γ-Terpinene, and p-Cymene. Furthermore, certain chemical components are found in both seeds and leaves, including Carvacrol, Caryophyllene, E-Caryophyllene, β-Bisabolene, cis-α-Bisabolene, Caryophyllene oxide, Z-Citral, E-Citral, γ-Terpinene, and δ-3-Carene (Farmanpour-Kalalagh et al., 2020). Dried summer savory consists of volatile oil, serving as a crucial source of chemicals such as carvacrol, thymol, and monoterpene hydrocarbons (beta-pinene, p-cymene, limonene, and camphene). Additionally, the leaves of summer savory contain vitamins and minerals. The findings from numerous studies indicate that various parts of summer savory exhibit a chemical composition that includes compounds such as Estragole, Carvacrol, E-Caryophyllene, Caryophyllene, γ-Terpinene, Thymol, p-Cymene, β-Bisabolene, cis-α-Bisabolene, Z-Citral, Caryophyllene oxide, E-Citral, and δ-3-Carene, as shown in Figure 7.

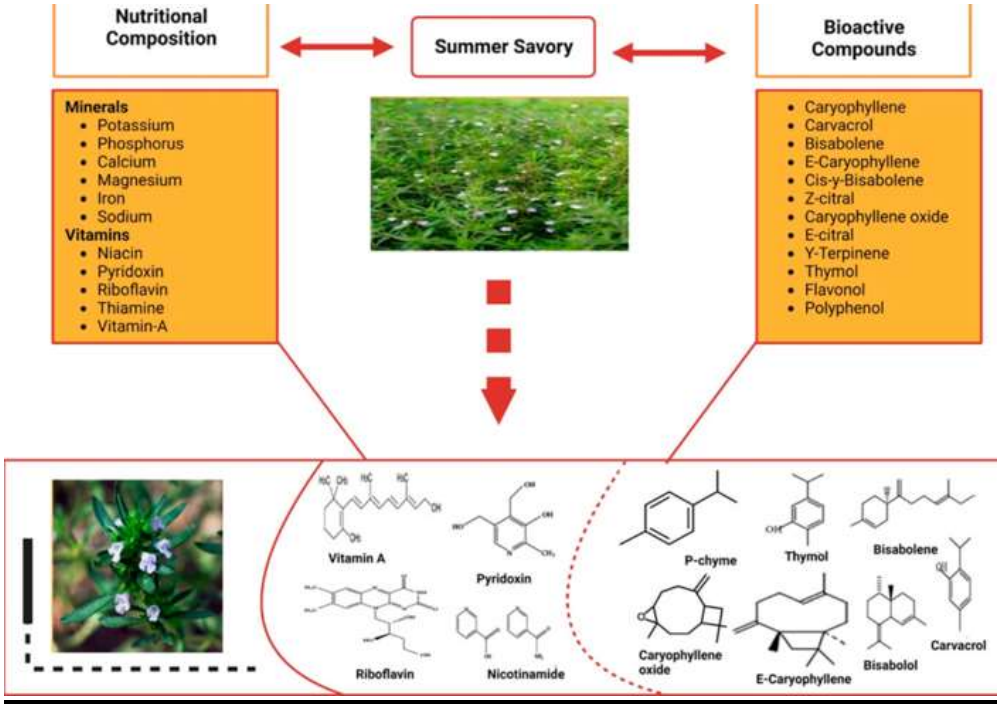



*Figure 7. Chemical Composition of Summer Savory (Ejaz et al., 2023)*

### 1.2.4 Medicinal Uses of Savory

Recently, various properties of Satureja species, including antimicrobial, antioxidant, anti-diabetic, anti-hyperlipidemic, antispasmodic, anti-nociceptive, anti-inflammatory, antiproliferative, sedative, reproduction stimulatory, and vasodilatory activities, have been substantiated by numerous studies (Bezić et al., 2009).

#### *1.2.4.1 Antioxidant Activity*

Research on various Satureja species has demonstrated their potent antioxidant properties. The essential oil extracted from the *S. hortensis* plant is abundant in isopropanoids like carvacrol, thymol, γ-terpinene, flavonoids, and other phenols, all of which are recognized for their robust antioxidant effects. Notably, the leaves of summer savory (*S. hortensis*) are particularly rich in phenolic compounds, including rosmarinic acid and flavonoids, contributing significantly to the high antioxidant capacity of the leaves (Bezić et al., 2009)

#### *1.2.4.2 Cancer*

Numerous investigations have established that phenolic acids and flavonoids possess diverse pharmacological properties, including antimicrobial, anti-inflammatory, vasoprotective, anti-carcinogenic, anti-allergic, and antiproliferative activities. Certain savory species, like *Satureja montana* L., have been employed in the treatment of several types of cancer. A report indicates that extracts from savory, particularly HeLa cells (human cervix epidermoid carcinoma), were found to be highly sensitive, with growth inhibition observed in HT-29 cells (human colon adenocarcinoma) at concentrations exceeding 0.7 mg/ml. These findings suggest that extracts from certain Satureja species, such as *S. montana* L., possess strong antioxidant properties and can selectively impede the growth of several human tumour cells (Cetojević-Simin et al., 2010) Carvacrol, an aromatic monoterpene present in the essential oils of numerous aromatic plants, has been studied for its impact on a human non-small cell lung cancer (NSCLC) cell line called A549. The research indicates that carvacrol exhibits an inhibitory effect on cancer cells while not significantly affecting normal lung cells (HFL1). These findings suggest that carvacrol may possess anti-carcinogenic properties and could potentially be utilized as a drug substance for cancer treatment (Koparal & Zeytinoglu, 2003).

#### *1.2.4.3 Antidiabetic and anti-cholesterol effects*

*Satureja khuzestanica* (SKE), a prevalent savory plant in Iran, is recognized for its antioxidant properties, and various studies have substantiated its anti-diabetic activity. SKE has been shown to reduce serum glucose and malondialdehyde levels in diabetic patients. Furthermore, the essential oil of SKE has been evaluated for its hepatoprotective, hypolipidemic, and antiatherogenic effects.

These properties could potentially decrease the risk of cardiovascular death and hepatic damage in individuals with diabetes (Ahmadvand et al., 2014).

#### *1.2.4.4 Cardiovascular Diseases*

Research studies have indicated that *Satureja hortensis* L. exhibits blood anticoagulant activity. Compounds such as carvacrol and other monoterpene hydrocarbons, flavonoids like apigenin, and phenolic acids like labiatic acid found in *S. hortensis* may contribute to its anti-platelet properties. Investigations have demonstrated that the methanol extract of *S. hortensis* L. has an inhibitory effect on blood platelet adhesion, aggregation, and secretion. This activity may provide a rationale for the traditional use of *S. hortensis* in treating cardiovascular and blood clot-related issues (Yazdanparast & Shahriyary, 2008).

#### *1.2.4.5 Alzheimer's Disease*

The deficiency of acetylcholine, a neurotransmitter, is widely accepted as a contributing factor to the development of Alzheimer's disease. Acetyl cholinesterase inhibitor drugs have been employed for the treatment of Alzheimer's, although many of these drugs are associated with side effects. The pursuit of natural compounds with antioxidant and anticholinesterase activities is considered highly desirable for managing this type of disorder (Öztürk, 2012). Antioxidants are recognized for their crucial role as neuroprotective agents in the early stages of Alzheimer's disease. A study indicates that certain Satureja species contain phenolic compounds, particularly flavonoids and flavonoid glycosides, which serve as potential sources of antioxidants. These antioxidants have the potential to slow down the progression of Alzheimer's disease and mitigate neuronal degeneration (Öztürk, 2012).

### 1.2.5 Savory in Animal Feed

*Satureja hortensis* is often integrated into feed formulations through methods such as dietary supplementation. For example, essential oils extracted from savory can be incorporated into the diets of poultry and swine at specified concentrations. Studies by Jamroz et al. (2006) demonstrated that the inclusion of savory essential oil in broiler diets positively influenced nutrient utilization and improved overall performance. Additionally, the antimicrobial properties of savory essential oil contributed to a reduction in pathogenic bacteria in the digestive tract, contributing to improved gut health. In the realm of ruminant nutrition, *Satureja hortensis* can be incorporated into forage-based diets or used as a supplement. Ruminants, including cattle and sheep, may derive advantages from the aromatic and palatable qualities of savory, potentially enhancing feed intake. Additionally, the bioactive compounds present in savory could play a role in modulating rumen fermentation, thereby improving nutrient absorption and overall digestive efficiency. The incorporation of *Satureja hortensis* into animal feed is influenced not only by the targeted animal species but also by specific

nutritional requirements and production goals. Whether utilized in monogastric diets for poultry and swine or included in ruminant or aquaculture feeds, the adaptability of *Satureja hortensis* underscores its versatility in various animal production systems.

## 1.3 Volatile Organic Compounds

Plants generate a diverse range of metabolites, but only a select few participate in "primary" metabolic pathways, which are shared by all organisms. On the other hand, a distinct set of metabolites, referred to as "secondary" metabolites, is unique to specific smaller groups of plants (Theis & Lerdau, 2003) These "secondary" metabolites arise as a result of various plant responses developed over the course of evolution to address specific needs (Pichersky & Gang, 2000). Within these metabolites, VOCs take center stage. Released by virtually all types of tissues and vegetation, including trees, shrubs, and grass, VOCs from plants come in various forms such as green leaf volatiles, nitrogen-containing compounds, and aromatic compounds. Plants can emit VOCs constitutively or in response to a range of stimuli (Holopainen et al., 2010). VOCs play a crucial role in a broad range of ecological functions, stemming from the interactions between plants and both biotic and abiotic factors. Plants utilize VOCs for diverse purposes, including indirect defence against insects, attraction of pollinators, communication between plants, adaptation to environmental stress and thermo-tolerance, as well as defence against predators (Baldwin et al., 2006). It's intriguing to note that the emission of VOCs is highly species dependent. Various plant lineages tend to employ distinct chemical solutions to address similar challenges. For instance, different flowers emit unique odorous volatiles to attract the same type of pollinator, a shared strategy despite the diverse plant species visited by these pollinators (Pichersky & Gang, 2000). According to the United States Environmental Protection Agency (EPA) volatile organic compounds are characterized by high vapor pressure and low water solubility (What Are Volatile Organic Compounds (VOCs)? | US EPA, n.d.). Categorized by their biosynthetic origin, VOCs can be grouped into three main categories: terpenoids, phenylpropanoids/benzenoids, and derivatives of FAs (Picazo-Aragonés et al., 2020) Terpenoids, constituting the most extensive family of VOCs, include over 550 compounds. These compounds originate from two shared five-carbon (C5) precursors: isopentenyl diphosphate (IPP) and its allylic isomer, dimethylallyl diphosphate (DMAPP) (Mcgarvey & Croteau', 1995). In plants, the C5-isoprene precursors essential for terpenoid synthesis are generated through two separate and isolated pathways: the mevalonic acid (MVA) pathway and the methylerythritol phosphate (MEP) pathway. The MEP pathway is exclusive to plastids, supplying precursors for hemiterpenes (C5), monoterpenes (C10), and diterpenes (C20). On the other hand, the MVA pathway is present in the cytosol, endoplasmic

reticulum, and peroxisomes, producing precursors for sesquiterpenes (C15) (Simkin et al., 2011).The second-largest category of plant VOCs is comprised of phenylpropanoids and benzenoids, originating from the aromatic amino acid phenylalanine (Phe) (Maeda & Dudareva, 2012). Within this VOCs class, there are three distinct subclasses distinguished by the structure of their carbon skeleton—phenylpropanoids (C6-C3 backbone), benzenoids (C6-C1), and phenylpropanoid-related compounds (C6-C2) (Muhlemann et al., 2014). The third significant category of VOCs comprises derivatives of fatty acids, including (Z)-3-hexenol, 2-ketones, and methyl jasmonate. These VOCs originate from C18 unsaturated FAs, specifically linoleic acids or linolenic acids (Picazo-Aragonés et al., 2020).

### 1.3.1 Volatile Organic Compounds in Animal Feed

Animals are often exposed to a diverse range of food sources containing plant VOCs, influencing both the flavor and nutritional content of animal-derived products. Foraging animals, relying on a diet that includes plants like alfalfa, clover, and grasses, experience the impact of VOCs on the aroma and taste of milk and meat (Kilcawley et al., 2018).The fermentation and drying processes involved in the production of silage and hay, crucial components of livestock feed, can affect the retention and release of VOCs in the final feed (Kung et al., 2018). Oilseed crops such as soybeans, canola, and sunflower, commonly used in animal feeds, may contain VOCs, particularly in their oil extracts (Nehmeh et al., 2022). The incorporation of essential oils from herbs, medicinal plants, and certain by-products into animal feeds introduces VOCs that could potentially offer health benefits to the animals (Ramdani et al., 2023). The intricate interplay of various factors related to the conditions of farmed animals significantly influences the quantities and types of VOCs present in animal-derived products. The composition of the animal's diet is a key determinant, as forages, grains, and supplements contribute specific compounds to the VOC profile of products like milk and meat. For instance, the aroma and flavour of these products can be shaped by the terpenes and other VOCs originating from different feed sources (Kilcawley et al., 2018). Livestock housing conditions, encompassing ventilation rates and air quality, play a pivotal role in determining VOC concentrations within animal spaces. Efficient ventilation systems can disperse and dilute VOCs, minimizing their buildup and reducing overall exposure for animals. Conversely, poor ventilation may lead to VOC accumulation, influencing animal exposure and potentially altering the VOC composition in derived products (Werth et al., 2014). Understanding the combined effects of these factors is essential for a comprehensive assessment of the VOC content in animal products, elucidating its potential implications for both animal health and the sensory characteristics of the final products.

### 1.3.2 Volatile Organic Compounds Effect on Meat

Meat and meat products serve as a significant protein source in the human diet, and their consumption is influenced by various factors such as socio-economic considerations, ethical considerations, religious beliefs, and cultural traditions (Font-i-Furnols & Guerrero, 2014). The flavour of meat is a crucial element of meat quality, playing a significant role in shaping consumer preferences and, consequently, holding high importance for the meat industry. A substantial portion of meat flavour is attributed to the presence of VOCs (Ni et al., 2022). This flavour develops through multifaceted reactions involving non-volatile precursors present in raw meat, influenced by temperature. 1,000 VOCs have been detected in meat and meat products, comprising predominantly of aldehydes, alcohols, ketones, acids, and various other compounds. Volatile compounds arise from Maillard reactions, lipid oxidation, interactions between products of Maillard reactions and lipid oxidation, as well as thiamine degradation. The resulting flavour is influenced by various factors, including the characteristics of the raw material (such as breed, sex, diet, and age of the animal, conditions and process of slaughter, duration and conditions of meat storage, and type of muscle), the use of additives, and the specifics of the technological process (Kosowska et al., 2017). Numerous research studies have indicated that particular individual VOCs can serve as biomarkers, supplying valuable insights into various aspects of meat, including the ability to distinguish between species, breeds, and the duration of aging (D. Liu et al., 2020). Furthermore, the profiles of VOCs can be employed to evaluate the quality of meat with certifications such as Protected Designation of Origin (PDO), Protected Geographical Indication (PGI), and Traditional Specialty Guaranteed (TSG). These profiles also hold promise as a dependable tool for detecting food adulteration (Legako et al., 2015). In a study conducted by Mancinelli et al. (2021) on raw and cooked meat samples derived from 4 strains of chicken differing in their growth rates, twenty primary VOCs were recognized and categorized into chemical groups such as aldehydes, alkenes, furans, amides, alcohols, and other compounds. The findings indicated that both chicken genotype and cooking method significantly impact the VOC composition of the meat (Cartoni Mancinelli et al., 2021). After the assessment of the meat's VOC composition through proton-transfer reaction–mass spectrometry (PTR–MS), and conducting a specific identification of VOCs using PTR–time of flight-MS (PTR-ToF-MS), it has been shown that the influence of cooking on VOC production was pronounced, with the VOC content detected in cooked samples being 5.5 times higher than that in raw meat. According to Figure 8, among the various compound groups identified, aldehydes stood out as the most prominent in cooked meat across all genotypes, constituting 64.85% of the total VOC. Within the aldehyde category, ethanal emerged as the primary compound. Alkenes, mainly

1,4-hexadiene (2.44%), along with furans, amides, and alcohols, were present in minor quantities as components of the detected VOC (Cartoni Mancinelli et al., 2021).

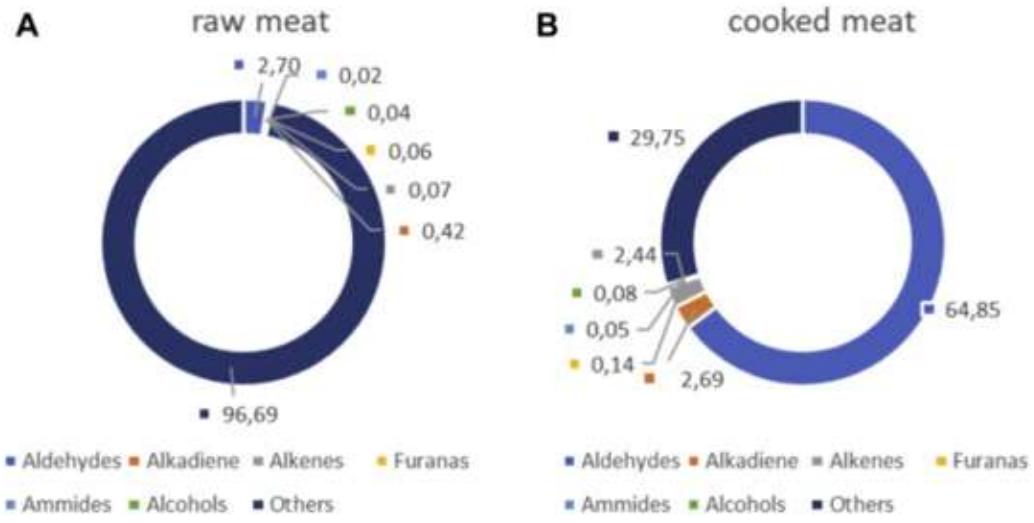



*Figure 8. . Main VOC classes (%) in raw (A) and cooked (B) chicken meat (https://www.sciencedirect.com/science/article/pii/S0032579120307835#tbl1).*

### 1.3.3 Volatile Organic Compounds in Milk and Milk Products

Milk and milk products cater to diverse consumer groups worldwide, offering a rich array of nutrients essential for human nutrition (Alegbeleye et al., 2018). The quality attributes of dairy products exhibit significant variability due to numerous factors that define their "identity," referring to specific compositional and sensory features intricately linked to the local environment (Natrella et al., 2020). Milk flavor is comprised of VOCs, which are crucial characteristics that consumers use to assess the quality of milk (Yuan et al., 2023).The VOCs present in milk and milk products originate from animal metabolism and their interaction with the surrounding environment, specifically influenced by factors such as diet and rearing conditions (Bergamaschi & Bittante, 2018). Based on a study conducted to assess mozzarella and milk samples from 22 dairy farms in the province of Bari using sensory analysis by 8 trained assessors belonging to the Italian Association of Cheese Tasters (ONAF), in addition to chemical analysis, the VOC profile and sensory attributes of milk and mozzarella were obtained. Forty-nine molecules were identified across the full set of samples, including five aldehydes, nine ketones, nineteen alcohols, one terpene, one aromatic hydrocarbon, one ester, nine acids, and three sulphur compounds. Remarkably, acetone emerged as the most abundant molecule, constituting approximately two-thirds of the total VOC concentration (Natrella et al., 2020). The prevalence of acetone as the dominant compound among all milk volatiles has been frequently documented in the literature, suggesting that it likely

plays a pivotal role in contributing to the aroma profile (Clarke et al., 2021). From a sensory perspective, only the milk samples were distinguishable by odour, indicating that processing immediately impacted the aroma of the final product. This distinction may be attributed to the fact that mozzarella, like all other "pasta-filata" cheeses, undergoes intense heating during the stretching phase, maintaining the curd temperature around 70°C for several minutes. This process is likely to result in the volatilization of numerous compounds and the formation of others that could overshadow the 'primary odours' of milk (Natrella et al., 2020).

### 1.3.4 Volatile Organic Compounds in Human Feed

In contemporary times, consumers exhibit a growing preference for food with authentic, natural, and delightful flavours, a preference that is intricately connected to the presence of VOCs in food (Gong et al., 2023). Plants generate VOCs through both primary and secondary metabolic pathways, constituting approximately 1% of all secondary metabolites. Over 1700 volatile chemicals have been identified across more than 90 gymnosperms and angiosperms. Despite the requirement for volatility, only a small fraction (5%–10%) of these molecules actually contribute to the overall aroma of food (Ma et al., 2022). Plant volatile organic compounds found in food have been associated with various health benefits, contributing to overall well-being. These bioactive compounds often exhibit antioxidant, anti-inflammatory, and antimicrobial properties, among other positive effects on human health. The first project in health and wellbeing of the University of Oxford concentrated on investigating how biogenic VOCs contribute to fostering beneficial health effects. Substantial evidence indicated that VOCs emitted by specific plant species have the potential to bring about physiological and psychological transformations that enhance various aspects of both physical and mental health. For instance, these compounds have been linked to a decrease in stress and anxiety levels, a reduction in stress hormone levels, and an increase in natural killer cells, collectively contributing to improved overall well-being (Leverhulme Centre for Nature Recovery | The Role of Volatile Organic Compounds in Physical and Mental Wellbeing Outcomes, n.d.).

### 1.3.5 The Future of Plant Volatile Organic Compounds (pVOCs) Research: Advances and Applications for Sustainable Agriculture

Addressing contemporary environmental challenges in agriculture demands immediate attention to develop new sustainable solutions. The intensification of productivity and the essential need for food security necessitate a sustainable approach to harnessing natural resources and metabolites. The agronomic potential of VOCs emitted from leaves represents a natural and eco-friendly solution for defending plants against various stresses and bolstering crop production. While the current use

of VOCs primarily focuses on combatting herbivores, we posit that their potential applications extend much further. VOCs have demonstrated capabilities in safeguarding plants against pathogens and environmental stresses. They play a pivotal role in priming plant defence mechanisms, enhancing resistance/tolerance to impending stress, neutralizing reactive oxygen species (ROS), exhibiting potent antimicrobial and allelopathic effects. Furthermore, VOCs may play a crucial role in regulating plant growth, development, and senescence through intricate interactions with plant hormones. This broad spectrum of applications underscores the versatility and untapped potential of VOCs in sustainable agricultural practices (Brilli et al., 2019). Volatile organic compounds serve as airborne signals, enabling rapid defence communication between distant plant organs (Heil & Bueno, 2007). Moreover, VOCs have the ability to "prime" the defence system of plants, leading to an augmented resistance against impending stresses (Conrath et al., 2002). Potential applications of plant VOCs in agriculture are manifold: Isoprenoids emitted by leaves possess the ability to provide protection against abiotic stressors, either by quenching ROS or by fortifying cell membranes. Certain VOCs exhibit the capacity to hinder the germination and growth of plant pathogens *in vitro*. VOCs can influence the plant's defensive system by stimulating the synthesis of defence proteins and metabolites, such as phytoalexins, which impede microbial colonization. Acting as priming stimuli, VOCs induce epigenetic changes and the accumulation of transcription factors, expediting the expression of plant defences for enhanced tolerance or resistance to future stress episodes. Additionally, VOCs can engage with the senescence mechanism or be harnessed to combat unwanted weed species through allelopathic effects (Brilli et al., 2019). In the present era, the advent of advanced analytical technologies, such as high-resolution Proton Transfer Reaction "Time-of-Flight" mass spectrometry (PTR-TOF-MS), enables the instantaneous and highly sensitive detection of the complete spectra of volatile organic compounds with exceptional resolving power (Graus et al., 2010) This technology allows for *in vivo*, comprehensive, and high-throughput measurement of the entire spectrum of VOCs, commonly referred to as the "volatilome," emitted from plant leaves. Phenotyping the volatilome has the potential to facilitate non-invasive screening of plant VOC profiles. This capability can be instrumental for breeders in selecting cultivars that demonstrate resilience under evolving environmental conditions and associated biotic stressors (Araus & Cairns, 2014). The application of PTR-TOF-MS analysis has the additional potential to facilitate a real-time diagnosis of crop health status. This involves monitoring specific VOC emissions in the air, such as MeSA (methyl salicylate) and sesquiterpenes, which act as stress biomarkers triggered by various abiotic and biotic constraints (Chalal et al., 2015). Furthermore, the temporal variations in VOC emission patterns can be leveraged for precision agriculture applications, enabling the monitoring of plant growth and development in the field. Like genomics and high-throughput platforms for

imaging and remote sensing, the real-time and highly resolved detection of VOCs generates vast amounts of data. The generation of 'big data' in this context necessitates computational analysis to extract patterns and identify features that are valuable for phenotyping (Singh et al., 2016). Utilizing machine learning tools for processing information on volatile organic compound emissions, in conjunction with environmental parameters gathered in the field by multiple sensors, enables the exploration of big data. This approach aims to measure plant performance and identify early symptoms of stress, thus leading to sustainable agriculture.

## 1.4 Fatty Acids in Milk

For centuries, milk has been recognized as the most complete natural nourishment (Park Young, 2009). Presently, it holds a significant position in the diets of more than 6 billion individuals worldwide (Haug et al., 2007). Bovine milk, in particular, accounts for the majority (83%) of global milk production. Over the past thirty years, this production has witnessed a substantial growth of approximately 60%, surging from 522 million tons to 828 million tons between 1987 and 2017. This expansion has been driven by the increasing need for dairy items to satisfy rising consumer demands. Bovine milk comprises the essential nutrients necessary for the calf's growth and maturation, constituting a reservoir of lipids, proteins, amino acids, vitamins, and minerals. It also contains immunoglobulins, hormones, growth factors, cytokines, nucleotides, peptides, polyamines, enzymes, and various other bioactive peptides. The lipids in milk are emulsified in globules coated with membranes. The proteins are in colloidal dispersions as micelles. The casein micelles occur as colloidal complexes of protein and salts, primarily calcium. Lactose and most of the minerals exist in a dissolved state within the milk. Milk composition has a dynamic nature, with its composition fluctuating based on factors like the stage of lactation, age, breed, nutrition, energy balance and health status of the udder (Cerbulis & Farrell, 1975). The FAs in milk are almost evenly sourced from two origins: the feed provided to the cow and the microbial activity that takes place in the cow's rumen (Parodi, 2004). The FA synthesis system in the mammary gland of the cow produces fatty acids with an even number of carbons ranging from 4 to 16 in carbon’s length. This synthesis accounts for approximately 60% on a molar basis and 45% on a weight basis of the total FAs. *De novo* synthesis in the mammary gland involves the production of C4:0–C14:0 acids and about half of the C16:0 fatty acid, which is derived from acetate and β-hydroxybutyrate. Acetate and butyric acid are generated in the rumen through the fermentation of feed components. Butyric acid is converted to β-hydroxybutyrate during absorption through the rumen epithelium (German & Dillard, 2006). Bovine fat also contains fatty acids with an odd number of carbons, such as pentadecanoic acid (C15:0) and heptadecanoic acid (C17:0), which are synthesized by the bacterial

flora in the rumen. The remaining C16:0 and long-chain fatty acids come from dietary lipids and the lipolysis of triacylglycerols in adipose tissue. Medium- and long-chain fatty acids, particularly C18:0, may undergo desaturation in the mammary gland to form the corresponding monounsaturated acids (Parodi, 2004).

### 1.4.1 Fatty acids Classes and Significance

The characteristics of milk, including its quality, processing capability, and sensory attributes, are closely linked to the content and composition of milk fat. The fat content in cow milk typically ranges from 3.3% to 4.4% (J Djordjevic1, 2019). Milk fat is comprised of a diverse array of lipid substances, with triglycerides (triacylglycerides) being the predominant component, constituting 98% of the total milk fat by weight. Other lipid compounds in milk include diacylglycerides (0.25-0.48%), monoacylglycerides (0.02-0.04%), phospholipids (0.6-1.0%), cholesterol (0.2-0.4%), glycolipids (0.006%), and free fatty acids (0.1-0.4%). The triclycerides in milk consist of over 400 different FAs, each possessing unique physico-chemical and biological properties (Joanna Barłowska, 2009). Biologically active lipid substances mainly consist of saturated fatty acids (SFAs), monounsaturated fatty acids (MUFAs; specifically linoleic acid; C18:2 n-6), and polyunsaturated fatty acids (PUFAs; particularly α-linolenic acid; C18:3 n-3). PUFAs with 20 carbon atoms, specifically docosahexaenoic acid (DHA; C20:5 n-3) and eicosapentaenoic acid (EPA; C22:6 n-3), serve as precursors for eicosanoids, which play a regulatory role in various physiological processes. The composition of FAs is influenced by factors such as animal species, breed, season, lactation stage, geographical location, and diet (J Djordjevic, 2019). Incorporating forage, particularly fresh grass, into the diets of dairy animals not only increases the proportion of unsaturated fatty acids in milk fat relative to SFA but also boosts the presence of conjugated linoleic acid. From a perspective of human health, the intake of SFAs, particularly lauric, myristic, and palmitic acids (C12:0, C14:0, C16:0, respectively), is associated with elevated levels of low-density lipoprotein (LDL) in the blood. However, it's noteworthy that other SFAs found in milk counteract this effect by increasing high-density lipoproteins (HDL) in the blood. In contrast, unsaturated fatty acids are generally considered beneficial for human health. Docosahexaenoic acid (DHA) serves as a crucial structural component in the brain, retina, and semen. Additionally, DHA plays a role in the development of the nervous system, vision processes, the growth of premature babies and children, and contributes to inflammation prevention (P.W. Parodi, 2004). Historical evidence suggests that maintaining a balanced n-6/n-3 fatty acid ratio of 1:1 was a significant factor in human evolution. However, the global shift from saturated animal fats to unsaturated plant fats and the widespread adoption of intensive milk and meat production, primarily through grain-based animal feeding, have

resulted in an increased intake of linoleic acid (LA), a precursor of the n-6 PUFA group. Due to competition between the n-6 PUFA group and the n-3 PUFA group for desaturation enzymes, the dietary ratio of n-6/n-3 fatty acids in modern human diets has been altered significantly, shifting from 1:1 to 10-20:1 or even higher. This shift may, in part, explain the emergence of modern diseases such as cardiovascular disease, cancer, obesity, and diabetes (Simopoulos, 2008).

#### *1.4.1.1 Saturated Fatty Acids (SFAs)*

Saturated fatty acids contain exclusively single bonds. Stearic, palmitic, myristic, lauric, caprylic, and butyric acid are instances of saturated fatty acids (SFA), with palmitic acid (C16:0) being the most prevalent. This group of fatty acids is predominantly present in matrices of animal origin, such as dairy products (butter, margarine, shortening) and meats (lamb, pork, and beef). Additionally, SFA can be also found in plant fats like coconut, cottonseed, and palm oils, as well as in lard and tallow (Echave et al., 2023) Saturated fatty acids constitute approximately 70% by weight of the total fatty acids present in milk. Palmitic acid (C16:0) is the most significant fatty acid in terms of quantity, constituting approximately 30% by weight of the total fatty acids. Myristic acid (C14:0) and stearic acid (C18:0) contribute 11% and 12% by weight, respectively. Among the saturated fatty acids, 10.9% are short-chain fatty acids (C4:0–C10:0). On an annual average, the proportions of butyric acid (C4:0) and caproic acid (C6:0) are 4.4% and 2.4% by weight of the total fatty acids, respectively in Swedish dairy milk (Lindmark Månsson, 2008).

##### *1.4.1.1.1 Functional Attributes of SFA*

Many phospholipids found in cell membranes contain substantial proportions of palmitic and stearic acids. Neural cell membrane phospholipids, in particular, may include longer chain SFAs. Ceramides and sphingolipids can be abundant in saturated fatty acids, whereas gangliosides often exhibit high levels of stearic acid. The elevated SFA content in these structures is associated with their membrane location and functional roles. Lipid rafts, microdomains in the plasma membrane serving as cell signalling platforms, typically contain phospholipids and sphingolipids rich in saturated fatty acids (Simons & Gerl, 2010). Myristic and palmitic acids have the capability to covalently modify various proteins participating in cell signalling. The fatty acylation serves a crucial role in anchoring these proteins to the inner leaflet of the plasma membrane, and it is also significant in facilitating protein trafficking between different organelles (Russell Johnson et al., 1994) Numerous saturated fatty acids with chain lengths ranging from 10 to 18 carbons have been documented to increase the levels of messenger RNA (mRNA) for PGC-1β, which acts as a coactivator for the sterol response element-binding protein (SREBP) family of transcription factors. SREBPs play a regulatory role in over 30 genes associated with the synthesis and metabolism of cholesterol, fatty acids, triacylglycerols, and phospholipids. This includes genes responsible for the

low-density lipoprotein (LDL) receptor, fatty acid synthase, and stearoyl-CoA desaturase (Xu et al., 2013). The impact of SFAs on blood coagulation and the counteracting process of fibrinolysis has been examined extensively. Various studies have explored the effects of saturated fatty acids in general or focused on individual ones. For instance, lauric, myristic, and palmitic acids, but not stearic or medium-chain saturated fatty acids, have all been shown to elevate fasting factor VII coagulant activity. Additionally, palmitic acid has been found to hinder the fibrinolytic activity of plasma. These observations imply that, in comparison to common unsaturated fatty acids, prevalent saturated fatty acids may contribute to a procoagulatory state (Calder, 2015). Extensive literature from both cell culture models and animal studies suggests that saturated fatty acids can induce insulin resistance and provoke a metabolic profile reminiscent of type 2 diabetes. Furthermore, evidence from human studies reinforces a positive association between the intake of dietary SFAs or the presence of circulating SFAs and the development of insulin resistance in humans (Risérus, 2008). *In vitro* and animal feeding studies indicate a potential role for SFAs, especially lauric and palmitic acids, in promoting inflammation. Numerous observational studies have explored the connection between exposure to SFAs (including dietary intake or circulating concentrations) and the presence of circulating markers of inflammation in humans. Some of these studies reveal a positive association between circulating SFAs and markers of inflammation, such as C-reactive protein (CRP) and interleukin (IL)–6 (Fernández-Real et al., 2003). There is substantial evidence suggesting that the primary dietary SFAs, including lauric, myristic, and palmitic acids, have the potential to elevate both total and LDL cholesterol levels. Additionally, these fatty acids have been linked to increased coagulation, the induction of insulin resistance, and the promotion of inflammation (Calder, 2015) The anticipation is that an elevated intake of SFAs will be linked to an increased risk of cardiovascular disease (CVD). A recent study delved into the connection between plasma phospholipid fatty acids and the occurrence of coronary heart disease (CHD). Data was collected from approximately 2400 men and women who developed CHD during the follow-up period and 4930 individuals who did not. The study found that higher levels of total saturated fatty acids, specifically palmitic acid and stearic acid (but not myristic acid), were associated with an increased risk of CHD (Khaw et al., 2012).

#### *1.4.1.2 Unsaturated Fatty Acids*

Unsaturated fatty acids are characterized by carbon chains that contain one or more double bonds along with a terminal carboxylic group (–COOH). This is in contrast to saturated fatty acids, which lack double bonds. These unsaturated fatty acids are further categorized into two groups based on the number of double bonds they possess. If there is a single double bond, they are termed

monounsaturated, while those with more than one double bond are referred to as polyunsaturated (Powell & Wallace, 2020).

#### *1.4.1.2.1 Monounsaturated Fatty Acids (MUFAs)*

About 25% of the FAs present in milk are MUFA, with oleic acid (C18:1) representing 23.8% by weight of the total FAs in Swedish dairy milk (Lindmark Månsson, 2008). Monounsaturated fatty acids are a significant component of the fatty acid profile in dairy milk, contributing to the nutritional quality and sensory attributes of the milk. The major MUFA found in dairy milk is oleic acid (C18:1), which plays a crucial role in the composition of milk fat. In addition to that, oleic acid is a monounsaturated Ω-9 fatty acid with a single double bond at the ninth carbon from the methyl end. Several scientific studies have investigated the specific types and concentrations of MUFAs in dairy milk. Research by Jensen RG (2002) provides a comprehensive overview of the fatty acid composition of bovine milk lipids, elucidating the prevalence of oleic acid among MUFAs (Jensen, 2002). In the milk of ruminants, there are additional contributions from other MUFA, albeit in smaller amounts yet with significant relevance. These include approximately 1% of 14:1, about 1.5% of 16:1, and a particularly desirable fatty acid known as vaccenic acid. Vaccenic acid serves as a precursor to conjugated linoleic acid (CLA) in the human organism, constituting a range of 1.5% to 5% (Markiewicz-Kęszycka et al., 2013).

##### *1.4.1.2.1.1 Functional Attributes of MUFAs*

Monounsaturated fatty acids do not lead to the buildup of cholesterol, a phenomenon associated with saturated fats, and they exhibit greater stability compared to polyunsaturated fatty acids, which are prone to becoming rancid. Additionally, monounsaturated fatty acids have a beneficial impact on the levels of high-density lipoproteins (HDL). HDL plays a crucial role in transporting cholesterol from the walls of blood vessels to the liver, where it undergoes degradation by bile acids before being excreted from the body (Markiewicz-Kęszycka et al., 2013). Therefore, they reduce the concentration of LDL, which when circulating over the entire organism are deposited in blood vessels. The intake of MUFA through the diet contributes to promoting a favourable blood lipid profile, regulating blood pressure, enhancing insulin sensitivity, and managing glucose levels. Additionally, recent compelling data propose a role for the preferential oxidation and metabolism of dietary MUFA, influencing body composition and potentially mitigating the risk of obesity. Growing evidence from epidemiological studies and human clinical trials consistently highlights the cardioprotective effects associated with MUFA content in dietary fat. As discussions persist regarding the optimal composition of fatty acids in the diet, the potential benefits of increasing MUFA consumption, especially as a substitute for dietary saturated fatty acids (SFA), warrant significant attention (Gillingham et al., 2011).

#### *1.4.1.2.2 Polyunsaturated Fatty Acids (PUFAs)*

Polyunsaturated fatty acids make up approximately 2.3% by weight of the total fatty acids in milk. The primary polyunsaturated fatty acids in this category are linoleic acid (C18:2) and alpha-linolenic acid (C18:3), which account for 1.6% and 0.7% by weight of the total fatty acids, respectively (Lindmark Månsson, 2008). Linoleic acid (C18:2 n − 6) and α-linolenic acid (C18:3 n − 3) constitute the primary polyunsaturated fatty acids (PUFA) in milk fat, with minor quantities of the long-chain PUFA, eicosapentaenoic acid (EPA; C20:5 n − 3), and docosahexaenoic acid (DHA; C22:6 n − 3) also detected. Linoleic acid (C18:2 n − 6) and α-linolenic acid (C18:3 n − 3) constitute the primary PUFA in milk fat, with minor quantities of the long-chain PUFA, eicosapentaenoic acid (EPA; 20:5 n − 3), and docosahexaenoic acid (DHA; 22:6 n − 3) also detected (Dewhurst et al., 2006).

##### *1.4.1.2.2.1 Functional Attributes of PUFAs*

The Expert Committee of the World Health Organization and the Food and Agriculture Organization has recommended maintaining a n-6/n-3 fatty acids ratio below 4. This specific ratio has shown a significant impact, leading to a substantial 70% reduction in the incidence of deaths attributed to cardiovascular diseases. Clinical study findings suggest that an elevated intake of n-3 FAs in the diet contributes to the prevention and treatment of various health conditions, including cancers, heart diseases, thrombosis, arterial hypertension, hyperlipidemia, senile dementia, Alzheimer's disease, depression, and rheumatoid arthritis. Additionally, n-3 fatty acids are employed in the therapeutic approach for certain skin diseases such as psoriasis, acne, and lupus erythematosus (McManus et al., 2011). Human cell membrane phospholipids consist of significant proportions of both linoleic acid and arachidonic acid. The proportions of these Ω-6 PUFAs can vary in cell membranes, and in certain cell types, arachidonic acid may contribute up to 20% of the fatty acids present. Linoleic acid plays a crucial role in the composition of ceramides, particularly those located in the skin. A deficiency in essential fatty acids can lead to a breakdown of skin integrity and an inability to prevent transdermal water loss. Maintaining an adequate intake of linoleic acid is essential to prevent essential fatty acid deficiency, with a relatively low intake requirement (1% of energy). Fortunately, most diets provide linoleic acid in quantities well exceeding this minimal requirement, ensuring the preservation of skin integrity and overall health (Rabionet et al., 2014). Extensive evidence indicates that linoleic acid effectively reduces blood cholesterol and LDL cholesterol concentrations, especially when it substitutes the prevalent saturated fatty acids. This cholesterol-lowering impact of linoleic acid is not a linear relationship across various intake levels, with the most significant effect observed when transitioning from a low to a moderate intake. Linoleic acid operates through Sterol Regulatory Element-Binding Proteins (SREBPs) to diminish cholesterol biosynthesis and enhance hepatic LDL receptor gene and protein expression. This

mechanism creates a favourable condition for the hepatic clearance of circulating LDL, contributing to the overall cholesterol-lowering effects of linoleic acid (Cho et al., 2002).

#### *1.4.1.3 Trans Fatty Acids*

Around 2.7% of the fatty acids present in milk are trans fatty acids containing one or more trans-double bonds. The primary trans C18:1 isomer is known as vaccenic acid (VA), specifically (C18:1 *t*11). However, trans double bonds in positions 4–16 are also detected in milk fat, albeit in minimal concentrations. Vaccenic acid makes up approximately 2.7% of the total fatty acid content and exhibits variations with the changing seasons (Precht & Molkentin, 1995).

### 1.4.2 Influence of Diet on Fatty Acid Composition

Considering that the FA composition of cow milk is influenced by the fatty acids derived from feed and the biohydrogenation process in the rumen, various factors come into play in shaping the fatty acid profile. These factors include the breed of the cow, the season, the lactation stage, the lactation number, the age of the dairy cows, the geographical location, and, notably, the diet. The diet stands out as the most crucial factor, accounting for approximately 95% of the variability in cow milk fat (Ellis et al., 2006). Lock and Garnsworthy (2003) demonstrated seasonal variability in the conjugated linoleic acid (CLA) content, assessed through Δ9-desaturase activity. Similarly, Elgersma et al. (2006) observed seasonal changes in the CLA content of cow milk, specifically noting differences between winter and summer. The fluctuations in the feed ratio between grass silage and fresh herbage throughout the seasons impact the fatty acid composition. This influence results in elevated levels of long-chain fatty acids (C17-C24), an increased ratio of unsaturated to saturated fatty acids, and reduced amounts of medium-chain fatty acids (C12-C16) (Frelich et al., 2009). The average content of rumenic acid in the milk of pasture-fed cows is two to three times higher than that in barn-fed cows (Jaroslav Blasko, 2010). The dietary intake of dairy cows, influencing microbiological processes in the rumen, and alterations in biohydrogenation processes, are pivotal factors in modifying the fatty acid composition of milk fat (Rennó et al., 2013). Lipid metabolism, particularly ruminal biohydrogenation, is impacted by various factors including rumen pH, as well as the quantity, source, and fatty acid profile of fat supplements in the diets of animals (Chilliard et al., 2001). The general trend indicates that milk fat content tends to increase with higher fibre content in various forages . The inclusion of forage, particularly fresh grass, contributes to an elevated proportion of unsaturated fatty acids in cow milk fat as compared to saturated fatty acids (Elgersma et al., 2006). Likewise, Chilliard et al. (2001) found that the levels of PUFA, particularly C18:3, C18:0, and C18:1, as well as SFA, especially C16:0, can be altered by increasing the inclusion of hay, fresh grass, and maize silage in the diet (Chilliard et al., 2001). In line with this,

other authors have proposed that the introduction of fat supplements in the diet leads to a more variable response in both milk production and composition compared to situations where diets are predominantly or entirely based on corn silage as the primary forage (Rennó et al., 2013). Conversely, diets with higher concentrate contents can offer substantial quantities of digestible carbohydrates while reducing the intake of fibrous components. This shift in dietary composition can lead to milk fat depression and alterations in the milk fatty acid profile (Z. L. Liu et al., 2008). A dairy cow diet supplemented with linseed oil, abundant in alpha-linolenic acid (ALA), results in an increase in PUFA in milk, particularly ALA and cis-9, trans-11 conjugated linoleic acid (CLA). Conversely, the addition of sunflower and fish oil to the diet increases the levels of vaccenic acid and cis-9, trans-11 CLA (Mach et al., 2013).

#### *1.4.2.1 Effect of Aromatic Plants Supplementation on Fatty Acid Composition*

Supplementing the diet of dairy cattle with phytochemicals derived from various herbal plants has demonstrated positive effects on rumen fermentation. This supplementation has been associated with increased milk yield and improved overall health in dairy cattle (Calsamiglia et al., 2007). According to a study done by Kholif et al. (2012) on twenty-eight lactating Damascus goats whose diets were supplemented by essential oils from aromatic plants such as garlic and cinnamon, the experimental additives led to elevated levels of ruminal volatile fatty acids and propionate proportions, accompanied by a reduction in ruminal acetate proportion and ammonia nitrogen concentration. The introduction of experimental additives resulted in a significant increase ($p<0.05$) in unsaturated fatty acids in milk, particularly C18:1 *c*9 and conjugated linoleic acids (CLA). Among the treatments, CIN treatments showed an increase in C18:3n3 and C18:3n6 (Ω-3 and Ω-6), surpassing the levels observed in other treatments. In summary, supplementing the ration of lactating goats with plant essential oils, particularly CIN oil, demonstrated positive effects on milk yield and milk protein. Additionally, this supplementation contributed to the improvement of healthy fatty acid levels, including conjugated linoleic acid (CLA) and Ω-3 in the milk. In another study conducted by (Cozma et al., 2015) aiming to assess the impact of dietary supplementation with hemp seed oil (HSO) on lipid metabolism, as indicated by changes in the plasma lipid profile, liver function, and concentrations of FA, cholesterol, and vitamin A in goat milk, the addition of hemp seed oil (HSO) to the diet significantly changed the fatty acid composition of the milk. The alterations in milk FA composition aligned with a reduction in the synthesis of saturated de novo synthesized FAs (C10:0-C16:0) and an elevation in concentrations of C4:0, C18:0, and PUFAs. Furthermore, supplementation with hemp seed oil (HSO) notably increased the concentrations of *c*9, *t*11CLA and C18:1*t* in milk fat. These findings suggest that HSO can be utilized to modify the

content of FAs in milk, potentially benefiting human health, without adversely affecting goat performance or health, except for a potential hyperlipidemic effect (Cozma et al., 2015).

# Chapter 2

# Objectives and aims

This research aimed at comprehensively investigating the presence of bioactive molecules, including VOCs and FAs, from dietary sources, specifically hemp (*Cannabis sativa* L.) and savory leaves (*Satureja hortensis* L.), to milk in dairy cows. Through experimental testing with six dairy cows at the "Lucio Toniolo" Experimental Farm of the University of Padua (Legnaro, Italy), we seeked to evaluate the impact of incorporating hemp leaves and savory into their standard silage-based diet on both the aromatic profile and fatty acid composition of the resulting milk. The study aimed at identifying, quantifying, and characterizing the VOCs and fatty acids transferred from these aromatic plant sources to milk. This integrated approach will provide a comprehensive understanding of how dietary intake influences both the flavour/aroma and nutritional aspects of milk, offering insights into potential benefits for human health.

# Chapter 3

# Materials and methods

## 3.1 Experimental Protocol

This comprehensive research, denoted as part of the BioAroma project (BIRD213117/21) and conducted in collaboration between the DAFNAE department of the University of Padua and the Edmund Mach Foundation (San Michele all'Adige, Trento, Italy), assessed the impact of aromatic plants, specifically hemp (*Cannabis sativa* L.) and summer savory (*Satureja hortensis* L.), on the dairy characteristics of milk and the quality of derived products in terms of both aromatic and nutraceutical properties. The selected plants, recognized for their strong aromatic profiles, are meant to transfer bioactive molecules, such as terpenes, to milk when consumed by lactating cows. This *in vivo* experiment involved six purebred Simmental dairy cows, housed at the "Lucio Toniolo" experimental stable of the University of Padua (Legnaro, Padua, Italy), and seeked to provide a comprehensive understanding of the effects of hemp and savory supplementation on the aromatic and nutritional properties of resulting dairy products. The utilization of these aromatic plants aligned with a circular economy approach, considering their therapeutic properties and potential benefits for both animal nutrition and the quality of milk and derived products. The study encompassed a detailed evaluation of the FA profile and VOCs in the milk, shedding light on the intricate interactions between the selected aromatic plants and the resulting dairy products. This research contributes to the broader BioAroma project, serving as a vital component in elucidating the multifaceted involvement of aromatic plants in enhancing the overall quality of dairy products.

## 3.2 Latin Square Experimental Design

The experimental design for this study employed a 3 × 3 Latin square framework to systematically administer three distinct diets to three groups of cows over successive periods (Table 4). This strategic arrangement ensured that all animals received each of the tested diets, facilitating the separation of the animal's inherent variability from the effects of the dietary treatments. This Latin square design not only enhanced the precision of the study but also minimized the required number of animals for experimentation. Each experimental group comprised two lactating cows, for a total of six cows under evaluation. Each of the three experimental periods spanned 14 days (Table 5), with six days dedicated to the incremental administration of hemp or savory. The test cows, characterized by an average of 1.3 calvings, a milk production ranging from 23.8 ± 4.8 kg/d, an age range of 30 to 46 months, and an average body condition score of 3.41, were individually housed in pens with permanent bedding (Table 6). The animals were divided into three groups, each consisting

of two subjects, contributing to a controlled and systematic assessment of the impact of hemp and savory supplementation on dairy characteristics.

*Table 4. Experimental 3 × 3 Latin Square Design*

| | **Period 1** | | | **Period 2** | | | **Period 3** | | |
|---|---|---|---|---|---|---|---|---|---|
| **Date** | from 13.10 to 26.10 | | | From 27.10 to 9.11 | | | From 1.11 to 23.11 | | |
| **Group** | A | B | C | A | B | C | A | B | C |
| **Diet** | Control (CTR) | Hemp (H) | Savory (S) | Hemp (H) | Savory (S) | Control (CTR) | Savory (S) | Control (CTR) | Hemp (H) |

*Table 5. Organization of each experimental period*

| | Experimental period | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| **Days** | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 | 13 | 14 |
| **Diet** | TMR | | | TMR + Hemp or Savory | | | | | | TMR | | | | |

*Table 6. Characteristics of dairy cows*

| **Experimental Group** | **A** | **A** | **B** | **B** | **C** | **C** | **Mean ± SD** |
|---|---|---|---|---|---|---|---|
| **Cow Code** | 91 | 103 | 105 | 101 | 100 | 96 | - |
| **Calving Order** | 2 | 1 | 1 | 1 | 1 | 2 | 1.3 ± 0.5 |
| **Age (months)** | 46.3 | 32.2 | 30.2 | 32.3 | 36.3 | 39.5 | 36.1 ± 6.0 |
| **BCS (Body Condition Score)** | 3.25 | 3.25 | 3.75 | 3.75 | 3.00 | 3.50 | 3.4 ± 0.3 |
| **Milk Production (kg/d)** | 25.94 | 23.11 | 24.01 | 21.50 | 17.51 | 30.77 | 23.8 ± 4.8 |

## 3.3 Total Mixed Ratio and Tested Feed Administration

The total mixed ratio (TMR) comprised a mixture of raw materials (Table 9), was distributed using a feed mixer wagon. The leaves, thawed the day before administration (at 08:00), were provided to the animals. To ensure leaf intake for testing, half of the TMR portion was mixed with the total quantity of thawed leaves and given to the animal. Subsequently, during the day, the remaining TMR was offered near the feeding rack. Daily consumption of TMR and the two tested feeds was recorded by weighing the administered quantities and residues in the trough the next day before unloading the fresh TMR. The feed intake was calculated daily as the difference between the dry matter distributed with the feed mixer wagon and the trough residue. The experiment was conducted

during the autumn season, spanning from October 10th to November 23rd. Over each two-week period, for the initial six days, the cows were gradually fed increasing amounts of an aromatic plant (Table 7), representing 0.2-2% of their daily dry matter intake. Subsequently, during the following eight days, the administration of the herbs was halted. The dosage of fresh material administered to the cow was determined taking in account the different moisture content, ensuring an equivalent quantity of dry matter. Both hemp and savory were stored frozen at -20°C, and just before administration, were defrosted at room temperature for 1 h and coarsely chopped using a bio-shredder. The cows were housed in individual pens for the entire experiment, with the rotation of herb administration every experimental period of 2 weeks, following the outlined scheme (Table 7). At the commencement of the test, boxes were labelled with signs denoting the assigned diet for each animal, and these signs were relocated according to the experimental design. Each cow was provided with a designated milk bin identified by the cow's unique identification number during the milking phase. Subsequently, the milk from cows subjected to the same treatment was segregated into separate storage tanks to prevent any inadvertent mixing, ensuring the avoidance of "cross-contamination" among the milk from different experimental groups. The cows were milked twice a day in the milking parlour, at 06:00 and 17:00. Throughout the three experimental periods, samples of 500 mL of homogenized milk were collected at each milking and immediately stored in the refrigerator at +4°C. Following the ARAV protocol (Associazione Regionale Allevatori Veneto), to obtain a single milk sample for analysis and eliminate the variability due to morning and evening milking, the milk collected the night before was mixed in equal parts by weight with the sample taken the next morning. In total, 756 milk samples were collected and analysed. Milk composition analyses were conducted in the dairy laboratory (Department DAFNAE, Legnaro, Padova, Italy). The results of the analyses were promptly provided by the operators, allowing a daily assessment of the situation for each individual cow involved in the experiment and fed with the different tested feeds. In the present thesis, are reported and discussed only the chemical analysis of milk samples collected at the pick of herbs administration (day 10 of each experimental period: 6 cows × 3 periods × 2 replications = 36 milk samples).

*Table 7. Test feeds administration within each experimental period.*

| Day | Week | Hemp, Kg tq/d | Savory Kg tq/d | Milk Sampling and analysis |
|---|---|---|---|---|
| | **First Week** | | | |
| **1** | Monday | | | Chemical |
| **2** | Tuesday | | | Chemical |
| **3** | Wednesday | | | Chemical |
| **4** | Thursday | 0.5 | 0.2 | Chemical, VOC |
| **5** | Friday | 0.5 | 0.2 | Chemical, VOC |
| **6** | Saturday | 1 | 0.4 | Chemical, VOC |
| **7** | Sunday | 1 | 0.4 | Chemical, VOC |
| | **Second Week** | | | |
| **8** | Monday | 1.5 | 0.8 | Chemical, VOC |
| **9** | Tuesday | 1.5 | 0.8 | Chemical, VOC |
| **10** | Wednesday | | | **Chemical, VOC and FA** |
| **11** | Thursday | | | Chemical |
| **12** | Friday | | | Chemical |
| **13** | Saturday | | | Chemical |
| **14** | Sunday | | | Chemical |

Chemical: Milk Chemical Analysis, VOC: Volatile Organic Compounds , FA: Fatty Acids

## 3.4 Agronomic and Chemical Characteristics of Hemp Leaves (*Cannabis sativa L.*)

The hemp leaves utilized in the study belong to the Futura 75 variety, a French monoecious strain well-suited for cultivation in Italian regions, particularly in mountainous areas, and specifically intended for fibre production (De Vita et al., 2022). The cultivation of this variety was authorized in Italy under the regulations outlined in Law 242 of December 2, 2016, which provides guidelines for cultivating *Cannabis sativa* L. for industrial purposes such as fibre and biomass. The cultivation took place at the "Centro Cereali e Colture Industriali" (CREA-CI) in Rovigo (Veneto, Italy), during the summer of 2022, characterized by an average temperature of 21.4°C. Manual harvesting involved the selection of plants, with subsequent separation of stems and leaves. The harvested leaves were stored at -20°C, thawed when needed, and utilized in the experimental tests.

*Table 8. Characteristics of Hemp Plants Before and After Leaf Separation*

| Harvest Date | Number of Plants | Plant Weight (kg) | Leaf Weight (kg) | Leaf Incidence (%) |
|---|---|---|---|---|
| **29.07.2022** | 1790 | 67.5 | 26.7 | 39.56 |
| **02.08.2022** | 1826 | 68.8 | 28.5 | 41.42 |
| **04.08.2022** | 1602 | 74.0 | 27.8 | 37.57 |
| **Total** | 5218 | 210.3 | 83.00 | 39.47 |

## 3.5 Agronomic and Chemical Characteristics of Savory Leaves *(Satureja hortensis L.)*

The savory leaves used in the experimental trial were sourced from a farm located in Teolo (Padua, Italy) at an altitude of 300 m. The transplant was carried out in 2016, with no pesticide treatments, fertilization, or irrigation applied. Weed removal was performed manually. After flowering in August, manual harvesting took place. Immediately following harvest, the savory branches were frozen and stored at -20°C until the time of administration. It's observed that the savory was harvested in the second week of September, coinciding with its balsamic period. This timing is typically advantageous, aligning with the plant's optimal stage. However, during this instance, environmental conditions, particularly an unusually dry season, caused the balsamic period to arrive earlier than expected. As a result, harvesting in September exceeded this period, potentially leading to oil and terpene loss. While harvesting at this time was necessary, it underscored the impact of environmental factors on agricultural practices.

## 3.6 Chemical Analyses

### 3.6.1 Chemical Analysis of TMR and Aromatic Plants

All analyses related to the botanical fractions in this study were conducted at the centralized chemical laboratory La.Chi. of the DAFNAE department located in Legnaro (University of Padua). The TMR was analysed for dry matter (DM), crude protein (CP), ether extract (EE), and crude fibre (CF) following AOAC methods (1995, 942.05, 954.01, 920.39). Additionally, fibre fractions (NDF, ADF, and ADL) were carried out according to the procedure outlined by Van Soest et al. (1991).

*Table 9. Formulation of the Diet*

| Ingredient | Kg of fresh ingredient |
|---|---|
| | |
| **Alfalfa forage** | 3.0 |
| **Grass hay** | 2.5 |
| **Soybean extraction meal** | 0.3 |
| **Energy mix[1]** | 5.8 |
| **Protein mix[2]** | 3.5 |
| **Corn germ[3]** | 0.3 |
| **Molasses** | 1.0 |
| **Grass Silage** | 7.5 |
| **Corn Silage** | 14.0 |
| **$H_2O$** | 5.0 |

*1 energy mix: corn, barley and sugar cane molasses.*
*2 protein mix: soybean extraction meal, extruded soybeans, sunflower seed flour and gluten free, corn semolina.*
*3 corn germ: mix of flax seeds and corn germ.*

*Table 10. Chemical Characteristics (% DM) of Diet and of Aromatic Plants Involved in the Test*

| | Diet | Hemp | Savory |
|---|---|---|---|
| **Dry matter (% DM[1])** | 58.00 | 91.98 | 89.60 |
| **Ash[2] (% D.M.)** | 7.52 | 13.88 | 7.2 |
| **CP[3] (% D.M.)** | 14.68 | 7.59 | 8.43 |
| **EE[4] (% D.M.)** | 3.41 | 7.12 | 5.41 |
| **CF[5] (% D.M.)** | 5.54pm | 2.40 | 35.31 |
| **NDF[6] (% D.M.)** | 41.44 | 30.49 | 60.54 |
| **ADF7 (% D.M.)** | 21.12 | 15.81 | 45.62 |
| **Gross ADL[8] (% D.M.)** | 4.11 | 5.29 | 5.79 |
| **AIA[9] (% D.M.)** | 0.55 | 0.09 | 0.29 |
| **Net ADL (% D.M.)** | 3.95 | 5.20 | 5.50 |

*1 DM: dry matter, 2 Ash: mineral substances, 3 CP: crude protein, 4 EE: ether extract, 5 CF: crude fibre*
*6 aNDF: neutral detergent fibre determined with heat-stable α-amylase and without sulfite (cellulose, hemicellulose, lignin) 7 ADF: acid detergent fibre (hemicelluloses, lignins) 8 ADL: acid detergent lignin ₉AIA: acid insoluble ash*

### 3.6.2 Milk Analysis

The chemical analysis of milk using the MilkoScan™ FT2 (Foss Electric A/S, Hillerød, Denmark) instrument has provided comprehensive insights into its composition. This advanced instrument, utilizing Fourier Transform Infrared Spectroscopy Technology, routinely measures key components such as fat, protein, lactose, casein, total solids, total solids without fat, and urea in milk.

*Table 11. Milk chemical composition*

| Variable | Diet treatment (TMR) | | | SE[1] | P-Value | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | Control | Hemp | Savory | | TRT | Period | Group | Cow | Day |
| **pH** | 6.58 | 6.59 | 6.58 | 0.005 | 0.330 | 0.012 | 0.117 | 0.00 | 0.026 |
| **Fat %** | 4.01 | 4.41 | 4.57 | 0.161 | 0.252 | 0.024 | 0.318 | 0.006 | 0.000 |
| **Protein %** | 3.68 | 3.60 | 3.78 | 0.043 | 0.184 | 0.348 | 0.117 | 0.000 | 0.858 |
| **Lactose %** | 4.94 | 4.93 | 4.89 | 0.023 | 0.461 | 0.152 | 0.702 | 0.000 | 0.003 |
| **Casein %** | 2.85 | 2.80 | 2.91 | 0.031 | 0.238 | 0.503 | 0.197 | 0.000 | 0.151 |
| **Fat/Protein** | 1.15 | 0.87 | 1.00 | 0.113 | 0.396 | 0.066 | 0.570 | 0.883 | 0.000 |
| **Total Solids (TS) %** | 13.06 | 1.35 | 13.61 | 0.136 | 0.200 | 0.023 | 0.561 | 0.000 | 0.000 |
| **TS without fat %** | 9.28 | 9.25 | 9.36 | 0.036 | 0.290 | 0.146 | 0.187 | 0.000 | 0.904 |
| **Urea mg/dL** | 30.2 | 30.8 | 29.4 | 1.53 | 0.828 | 0.034 | 0.197 | 0.000 | 0.602 |

[1]SE: Standard Error

### 3.6.3 Milk sampling

According to the experimental design, 36 milk samples (2 milk samples × 6 cows × 3 experimental periods) were collected the day after the maximum administration of tested feeds to be analysed for VOCs and FA profile. For VOCs, 10 ml of milk samples were stored at +4°C to be immediately analysed, while for FA and chemical analysis, milk samples were stored at -20°C and then freeze-dried, ensuring the stability of the components for subsequent analyses. From the freeze-dried samples, approximately 100 mg of each was meticulously processed for further investigations.

### 3.6.4 Volatile organic compounds (VOCs) analysis with Headspace Solid-Phase Microextraction – Gas Chromatography-Mass Spectrometry (HS-SPME – GC-MS)

The chemical analysis conducted to determine the aromatic profile of milk and foods involves three essential stages. First was the extraction of VOCs through the headspace solid-phase microextraction method (HS-SPME). Subsequently, gas chromatography was employed for the separation of volatile compounds, followed by the last phase of mass spectrometry, facilitating the detection of VOCs. The entire analysis procedure was automated, ensuring independence and

accuracy, starting from the measurement and insertion of the sample into vials, up to the placement of the latter in the designated instrument site.

#### *3.6.4.1 HS-SPME Extraction*

The initial step involved the solid-phase microextraction of the headspace, and the automation of this stage is pivotal as it enables the mechanization of subsequent phases, namely gas chromatography and mass spectrometry. The automation process was facilitated through the utilization of the multipurpose automatic sampler (MPS GERSTEL). This instrument mechanizes the holder, analogous to a syringe, forming the foundation of the solid-phase microextraction process of the headspace. The core of this device is the extracting fibre, composed of fused silica fibre combined with a small quantity of the extracting phase. This material facilitates the extraction of volatile compounds from the sample, whether it is in a solid or liquid state. In the experiment, a three-phase SUPELCO SPME fibre (DVB/CAR/PDMS) with a length of 2 cm was employed for the extraction process. The matrices subjected to analysis included milk and various foods such as TMR, hemp, and savory. The samples, each with a predetermined size, were contained within vials: 8 ml for milk, with the addition of 2g of anhydrous sodium sulfate to facilitate the release of VOCs, and 300 mg for food samples. Once prepared, the vials were sequentially placed in the designated instrument site for analysis. The holder, part of the automatic sampling system, operates independently. The extracting fibre was extracted from the needle and exposed to the sample by being inserted through the upper part of the vial into its headspace—an area not occupied by the sample. In this headspace, volatile compounds are released, absorbed by the extracting phase (a part of the fibre). Following the exposure phase, the fibre is retracted, covered, and protected by the needle, preserving it until the desorption phase occurs within the gas chromatographic injector.

Prior to fibre use, a thermal preconditioning process lasting 25 minutes is employed to ensure optimal extraction activity. Subsequently, other phases of the extraction process, each with a known duration, can be carried out: the fibre exposure phase lasted 20 minutes, while the desorption (injection) phase lasted 3 minutes.

#### *3.6.4.2 Gas Chromatography Coupled to Mass Spectrometry (GC-MS)*

Following the HS-SPME extraction, the next phase involved the separation of the extracted compounds through a desorption phase followed by gas chromatography (GC) coupled with mass spectrometry (MS), enabling the identification and quantification of the compounds. The desorption phase occured at the injector level of the gas chromatograph (3 minutes) where, due to the temperature effect (250°C), VOCs were released by the absorbent phase of the fibre. Subsequently, the gas chromatographic run begun, lasting a total of 52 minutes. The initial temperature of the gas chromatography column Agilent 19091S-433 (Agilent Technologies, Santa Clara, California,

United States is set at 40°C for 1 minute, followed by a gradual increase to 160°C at 3°C/min and an immediate increase to 250°C at 10°C/min, maintained for 2 minutes. The flow of the gas chromatographic column was calibrated to 0.68445 mL/min. Inside the gas chromatographic column, volatile organic compounds ran at different speeds based on their molecular weight and chemical nature, influenced by the interaction with the stationary phase. The physical interaction allowed the separation of compounds based on molecular weight and chemical nature. The separated molecules reach the detector, a mass spectrometer in this analysis, which provides data on the molecular ion weight, fragments, abundance, and weight of the substances. The mass spectrometer generated a chromatogram, a graph processed by software, depicting signal intensity (y) representing peak height and retention time (x) determining the distance and distribution of peaks on the x-axis. Peaks indicate detected substances, while a flat baseline suggests no detection. The final step involved identifying different molecules by comparing their retention times with databases using software. The system provides information such as the name, retention time, and "match factor" indicating the affinity between the detected molecule and its counterpart in the comparison.

### 3.6.5 Lipid Extraction and Fatty Acid Methylation

The analytical exploration of the milk's fatty acid profile in this study encompassed distinct procedural steps, ensuring a comprehensive understanding. Initial sample preparation involved meticulous handling, followed by lipid extraction and esterification, where fats were efficiently processed to create fatty acid methyl esters (FAME). The subsequent phase involved the separation of these FAME components, utilizing the two-dimensional Gas Chromatography (GC×GC) technique. The GC×GC separation significantly enhances resolution compared to traditional one-dimensional GC, enabling a more detailed examination of complex samples. This methodological advancement proves instrumental in capturing intricate details of the milk's fatty acid composition. Each step, from sample preparation to the determination of the fatty acid profile, contributed to the precision and thoroughness of the analytical process. The methylation process, specifically transesterification to produce FAME (Fatty Acid Methyl Esters), was a crucial step in the analysis of fatty acids before GC×GC (Comprehensive Two-Dimensional Gas Chromatography). This process was essential for converting complex and simple forms of FAs into their methyl ester derivatives. The main purpose of this transesterification/methylation step was to enhance the volatility and chromatographic behaviour of fatty acids, making them suitable for analysis by GC. There are two commonly used catalysts for transesterification: base catalysts and acid catalysts. Base catalysts are known to avoid migration and isomerization of double bonds but may not effectively esterify free fatty acids. On the other hand, acid catalysis can esterify both free and

complex forms of fatty acids but may cause isomerization of conjugated double bonds (Kramer et al., 1997). The transesterification process, particularly the method proposed by Sukhija and Palmquist (1988) and modified by Jenkins (2010), involves direct acid-alkaline transesterification/methylation of freeze-dried samples. This method exposes the samples to high temperatures and acid-alkaline digestion for short durations. The goal is to achieve efficient conversion of fatty acids into their methyl ester forms while preserving the integrity of certain constituents, such as conjugated linoleic acid (CLA). By converting fatty acids into FAME, the resulting derivatives were more volatile and amenable to separation and detection by gas chromatography, facilitating a detailed analysis of the fatty acid profile in complex samples. The preparation of milk samples for methylation and esterification involved several key steps to convert the fatty acids into their methyl ester derivatives (FAME). The milk was first ground and allowed to equilibrate at room temperature for approximately 10 minutes. Subsequently, 100 mg of the ground milk was carefully measured and placed into culture tubes. Each sample in the culture tubes was then treated with 1 mL of sodium methoxide (NaOMe) (0.5M). The samples underwent an incubation process in a water bath set at 50 °C for 10 minutes. Following this initial incubation, the tubes were removed from the water bath and allowed to cool for 5 minutes. The next step involved the addition of 1.5 mL of freshly prepared methanolic HCl (1.37 M) to each sample. The samples were then subjected to a second incubation in a water bath, this time at 74 °C for 10 minutes. Afterward, the tubes were once again removed from the water bath and allowed to cool for 7 minutes. To further the esterification process, 2.5 mL of a potassium carbonate (K2CO3) solution (0.43M) and hexane ($C_6H_{14}$) were added to each tube. In order to account for variations in the fat content among samples, the volume of hexane added was adjusted accordingly. Specifically, 2 mL of hexane was added for every 40 mg of fat present in the sample. As the fat content varied across samples, the volume of hexane ranged from 1.5 mL to 3.5 mL as shown in (Table 12) to ensure proportional extraction. Hexane is added in the process to extract the FAMEs from the reaction mixture. Hexane is a nonpolar solvent that is particularly effective for extracting nonpolar compounds like FAMEs. After then, the contents of the tubes were vortexed for 30 seconds to ensure thorough mixing, and subsequently, the tubes underwent centrifugation for 10 minutes at 2000g and 10 °C. This centrifugation step helped in separating the phases, and the upper organic layer, containing the FAME, was transferred to GC vials for further analysis by gas chromatography.

*Table 12. Hexane Volume and Fat Content Variations in Milk Samples*

| Date | Group | Cow | Hexane (mL) | Fat Content (mg) |
|---|---|---|---|---|
| | A | 91 | 2.6 | 52 |
| | | 103 | 2.0 | 40 |
| **19/10** | B | 105 | 3.1 | 62 |
| | | 101 | 2.2 | 44 |
| | C | 100 | 2.8 | 56 |
| | | 96 | 2.8 | 56 |
| **02/11** | A | 91 | 2.6 | 52 |
| | | 103 | 2.0 | 40 |
| **02/11** | B | 105 | 2.4 | 48 |
| | | 101 | 2.4 | 48 |
| | C | 100 | 1.9 | 38 |
| | | 96 | 1.5 | 30 |
| | A | 91 | 2.5 | 50 |
| | | 103 | 2.4 | 48 |
| **16/11** | B | 105 | 3.5 | 70 |
| | | 101 | 2.4 | 48 |
| | C | 100 | 2.2 | 44 |
| | | 96 | 2.6 | 52 |

### 3.6.6 Fatty acid profile analysis with Two-Dimensional Gas-Chromatography (GC×GC)

Two-dimensional gas chromatography (GC×GC), a comprehensive multidimensional technique that has garnered recent interest, particularly in the context of food analysis, presents a potential alternative capable of addressing certain constraints inherent in FAME analysis. This is attributed to a substantial enhancement in separation power compared to one-dimensional GC. The utilization of two columns facilitates the physical separation of compounds within intricate and challenging samples (Tranchida et al., 2013). The FAME solutions obtained with the extraction method were analyzed for their FA profiles using a GC×GC instrument (7890A; Agilent Technologies, Santa Clara, CA, USA) with two columns in series, and equipped with a modulator (G3486A CFT; Agilent Technologies), an automatic sampler (7693A; Agilent Technologies) and a flame ionization detector (FID) connected to the Agilent Chemstation chromatography software (Agilent Technologies). Between the two columns, a modulator unit collected the bands of the first column in a fixed-

volume channel then launched them into the shorter second column in narrow bands. The operating conditions of the GC apparatus were as follows: the first column, 75 m × 180 mm (internal diameter) × 0.14 mm film thickness (23348U (polar); Supelco, Bellefonte, PA, USA); the initial H2 carrier flow of 0.2 mL/min was increased to 0.3 mL/min at a rate of 0.002 mL/min. The second nonpolar column, 3.5m × 250mm (internal diameter) × 0.14mm film thickness (J&W 19091-L431, Agilent Technologies); in this case the initial $H_2$ carrier flow of 22 mL/min was held for 2 min then increased to 30 mL/min at a rate of 0.08 mL/min. The initial oven temperature was 50°C, where it was held for 2 min; it was then increased at a rate of 2°C/min to 150°C, where it was held for 15 min, then increased at a rate of 2°C/min to 240°C, where it was held for 20 min. The valves were set to a modulation delay of 1 min, a modulation period of 2.9s, and a sample time of 2.77s. The gas flows consisted of hydrogen (20 mL/min) and air (450 mL/min). Each 0.8-mL sample was injected in the pulsed split mode at a pressure of 0.172 MPa _ 0.3 min and a split ratio of 150:1. The splitless inlet was run at a temperature of 270°C and a pressure of 20.80 MPa, with the septum purge set to 3 mL/min and a split flow of 35.2 mL/min. The resulting two-dimensional chromatograms were analyzed with comprehensive GC×GC software (GC Image R 2.2 GC×GC: Zoex Corp., Houston, TX, USA), and the cone volume determined for each FA.

## 3.7 Computational Procedures and Statistical Analysis

### 3.7.1 Identification and Quantification of Fatty acids

FA identification was accomplished through two distinct methods. Firstly, it involved comparing the cone positions in the chromatogram with those of FAs found in GC reference standards. These standards comprised mixtures of pure FAs such as #674, #463 (Nu-Chek Prep Inc.), 47080-U bacterial acid methyl esters (Sigma-Aldrich, St. Louis, MO), and 47085-U PUFA-3 menhaden oil (Supelco). Individual CLA isomers, including CLA c9,t11 (#UC-60M, Nu-Chek Prep Inc.), CLA t10,c12 (#UC-61M, Nu-Chek Prep Inc.), CLA c9,c11 (#1256, Matreya LLC, Pleasant Gap, PA), CLA t9,t11 (#1257, Matreya LLC), and CLA c11,t13 (#1259, Matreya LLC), were also part of these standards. The second procedure for FA identification involved assessing the elution order and position of each cone within the 2-dimensional chromatogram. This was achieved by utilizing comprehensive GC × GC software (GC Imaging Software, Zoex Corp.). Subsequently, during data analysis, specific challenges were addressed, including discrepancies in mass. This was observed as an additional unidentified cone in some FA peaks, and to rectify this, the mass loss value was incorporated into the respective FA mass. In addition to elucidating instances where overlapping isomers appeared, it is crucial to consider the underlying factors that may have contributed to this occurrence. Isomer overlapping in chromatographic analyses can arise from various sources,

including similarities in retention times and structural similarities between fatty acids. In my study, the complexity of the sample matrix, potential co-elution of closely related isomers, and the intricacies of the chromatographic system could have led to overlapping peaks. To address this challenge, a systematic approach involving comparisons with reference standards was implemented. Each overlapping isomer was scrutinized, and corrective measures were taken to discern and allocate the correct values. This meticulous process involved eliminating the contribution of the isomer not originally present in the reference standard and reallocating it to the appropriate isomer. These corrective actions were imperative for ensuring the precision and reliability of fatty acid identification and quantification in the face of overlapping isomers. Moreover, fatty acids present in exceedingly minimal and trace amounts, falling below the threshold of consideration for meaningful analysis, were judiciously eliminated from the study. The quantification of each fatty acid involved evaluating the cone volume of individual fatty acid peaks relative to the total fatty acid volume. The results were expressed as grams of a specific fatty acid per grams of total fatty acids, multiplied by 100. This calculation was based on the concentration of methyl 12-tridecenoate in the solution, serving as the internal standard. The cumulative amounts of different fatty acids were categorized as follows:

- SFA = sum (C4:0, C6:0, C8:0, C10:0, C12:0, C14:0, C16:0, C18:0, C20:0, and C22:0, odd-chain saturated fatty acids C5:0, C7:0, C9:0, C11:0, C13:0, C15:0, C17:0, C19:0, C21:0, branched-chain saturated fatty acids C13:0 iso, C14:0 *iso*, C15:0 *iso*, C15:0 *anteiso*, C16:0 *iso*, C17:0 *iso*, C17:0 *anteiso*, C18:0 *iso*, and C19:0 *anteiso*).
- MUFA= sum (10:1, Σ12:1 cis, 14:1 *c*9, Σ14:1$_{others}$, Σ15:1, Σ16:1, 17:1 *c*10, Σ17:1$_{others}$, 18:1 *c*9, 18:1 *c*11, 18:1 *t*11, 18:1 *c*15, Σ18:1$_{others}$, 20:1 *c*8, and 20:1 *c*11).
- PUFA = sum (18:2 *t*, 18:2 *c*, 18:2$_{others}$, 18:3, and C20-24)
- In the analytical framework of this study, the unsaturation index was determined by calculating specific ratios according to (Pegolo et al., 2016) for selected fatty acid pairs, namely C10:1/ (C10:0 + C10:1), C14:1/(C14:0 + C14:1), C16:1/(C16:0 + C16:1), and C18:1/(C18:0 + C18:1). This index, derived from the concentration of MUFA relative to the combined sum of SFA and MUFA, served as a quantitative measure to assess the degree of unsaturation in the lipid composition.

### 3.7.2 Statistical Analysis

The FA profile and VOCs were subject to statistical analysis using the PROC MIXED procedure of SAS (2007, version 9.4).

The model considered the following fixed effects and used the triple interaction of period, treatment, and group (Ti * Pj * Gk) as error line:

$y_{ilk} = \mu + T_i + P_j + G_k + V_l(G_k) + e_{ijkl}$

where:

y ijk = dependent variable;

μ = overall intercept of the model;

Ti = effect of treatment feed (i = 1, 2, 3)

Pj = effect of experimental period (j = 1, 2, 3)

G= effect of animal group (k = 1, 2, 3)

V= effect of cow within animal group (l = 1, 2)

e = random residual error N ~ (0, σ2e, 2 d.f.)

To test the effect of the experimental treatment, the following contrasts were evaluated:

1. Savory effect: Control vs. Savory (CTR vs S)
2. Hemp effect: Control vs. Hemp (CTR vs H)
3. Hemp vs. Savory: (H vs S)

The effects of the model were declared significant at $P < 0.10$ using the Bonferroni statement at $\alpha = 0.10$.

# Chapter 4

# Results and Discussion

The investigation into the volatile organic compounds (VOCs) excreted by cows fed with diets enriched with hemp *(Cannabis sativa L.)* and summer savory *(Satureja hortensis L.)* has revealed a diverse array of chemical constituents. The analysis allows the identification of 29 terpenes and 87 fatty acids. Terpenes derive from the right transfer of these compounds from the diet to milk while the fatty acids are the result of a complex digestive and metabolic process in response to the unique dietary components. In this exploration, the terpenes are anticipated to unveil the nuanced aromatic profile of the consumed botanicals, while the fatty acid composition will shed light on the nutritional aspects of the administered diets. This multidimensional analysis sets the stage for a detailed investigation into the specific terpenes and fatty acid profiles, allowing for a deeper comprehension of the physiological implications of these dietary interventions on the VOCs and lipid metabolism in the studied bovine subjects.

## 4.1. Terpenes Found in the Analysis and their Reference Sources

In Table 13, the thesis outlined the identification of 29 terpenes, presenting both their common names, commonly used in scientific literature, and their IUPAC names. The term "reference sources" referred to the limited information available regarding the families or plant species where these terpenes are typically found. What's noteworthy is that these terpenes aren't confined to aromatic plants but are also present in specific feeds constituting the cow's diet. Sunflower seeds and soy are highlighted as key components of the bovine diet, containing terpenes such as β-pinene, camphene, α-pinene, and limonene in sunflower seeds (Bocci & Frega, 1996), and β-myrcene in soybeans (Kim et al., 2020). This connection between the terpenes identified in the analysed samples, particularly in cow milk, and their presence in the diet, especially in sunflower seeds and soy, implies a potential relationship between the animals' diet and the composition of terpenes in their milk. Table 14 shows other molecules not classified as terpene but can be considered as relevant VOCs of the TMR and belong to the category of alcohols and aldehydes. The analysis of the HS-SPME-GC-MS found the presence of 20 alcohols and 17 aldehydes of which 2 major ones Vinyl Amyl Carbinol and Phenylacetaldehyde. The aldehyde identified is present in or characterize the feeds that make up the diet of dairy cows such as sunflower. In addition to that, the presence of alcohols in this matrix is not surprising as they are typical products of fermentations together with acids, aldehydes, and ketones carried out by the microflora which acidify the ensiled biomass and ferment the diet in the rumen. The presence of alcohols in the diet of dairy farmers can be traced back above all to corn, to sunflower (Vinyl Amyl Carbinol) and soybean

*Table 13. Terpenes Identified With HS-SPME/GC-SM Analysis*

| Common Name | IUPAC Name |
|---|---|
| **α-Thujene** | Bicyclo[3.1.0]hex-2-ene, 2-methyl-5-(1-methylethyl)- |
| **β-pinene** | β-Pinene |
| **β-myrcene** | β-Myrcene |
| **β-Citronellene** | 1,6-Octadiene, 3,7-dimethyl-, (S)- |
| **Camphene** | Camphene |
| **α-pinene** | α-Pinene |
| **δ-3-carene** | 3-Hulls |
| **o-cymene** | Benzene, 1-methyl-2-(1-methylethyl)- |
| **Limonene** | 1,2 D-Limonene |
| **δ-sylvestrene** | Cyclohexene, 1-methyl-5-(1-methylethenyl)-, (R)- |
| **trans-β-ocimene** | 1,3,6-Octatriene, 3,7-dimethyl-, (E)- |
| **terpinen-4-ol** | 3-Cyclohexen-1-ol, 4-methyl-1-(1-methylethyl)-, (R)- |
| **α-Phellandrene** | α-Phellandrene |
| **cis-Sabinene hydrate** | Bicyclo[3.1.0]hexan-2-ol, 2-methyl-5-(1-methylethyl)-, (1α.,2.β.,5.α.)- |
| **Linalool** | 1,6-Octadien-3-ol, 3,7-dimethylcis- |
| **β-ocimene** | 1,3,6-Octatriene, 3,7-dimethyl-, (Z)- |
| **α-Ylangene** | Ylangene |
| **β-cis-caryophyllene** | Bicyclo[7.2.0]undec-4-ene, 4,11,11-trimethyl-8-methylene-, [1R-(1R*,4Z,9S*)]- |
| **α-Zingiberene** | 1,3-Cyclohexadiene, 5-(1,5-dimethyl-4-hexenyl)-2-methyl-, [S-(R*,S*)]- |
| **cis,trans-α-Farnesene** | 1,3,6,10-Dodecatetraene, 3,7,11-trimethyl-, (Z,E)- |
| **β-caryophyllene** | Caryophyllene |
| **trans-α-bergamotene** | Bicyclo[3.1.1]hept-2-ene, 2,6-dimethyl-6-(4-methyl-3-pentenyl)- |
| **α-caryophyllene** | α-Caryophyllene |
| **β-Farnesene** | β-Farnesene |
| **α-terpineol** | 3-Cyclohexene-1-methanol, α,4-trimethyl- |
| **Camphor** | Bicyclo[2.2.1]heptan-2-one, 1,7,7-trimethyl-, (1R)- |
| **Thymoquinone** | 2,5-Cyclohexadiene-1,4-dione, 2-methyl-5-(1-methylethyl)- |
| **α-Citral** | 2,6-Octadienal, 3,7-dimethyl-, (E)- |
| **Caryophylladienol II** | Bicyclo[7.2.0]undecane, 10,10-dimethyl-2,6-bis(methylene)-, [1S-(1R*,9S*)]- |

*Table 14. Other Compounds Identified With HS-SPME/GC-SM Analysis*

| Common Name | IUPAC Name |
|---|---|
| **Vinyl amyl carbinol** | 1-Octen-3-ol |
| **Phenylacetaldehyde** | Benzeneacetaldehyde |

## 4.2. Quantitative Evaluation of Aromatic Compounds in Dietary, Hemp, and Savory Matrices: Analysing Concentration via Literature References

The examination of VOCs goes beyond identification, extending to a quantitative study of their abundance in diet, hemp, and savory matrices. The discussion was organized by molecule families, with a focus on terpenes, revealing aromatic profiles in these matrices. Our goal was to unveil not just the presence but also the quantitative distinctions of these compounds in dietary, hemp, and savory samples, contributing to a comprehensive understanding of aromatic composition in these investigated feed sources, drawing upon scientific references for validation.

### 4.2.1 Quantitative Evaluation of Aromatic Compounds in Hemp: Analysing Concentration via Literature References

The aromatic analysis of hemp involved the identification and quantification of 29 terpenes, revealing that 23 of these compounds corresponded with the aromatic profile of hemp reported in four scientific publications referenced as “e,f,g,h” in Table 14 (Giovannoni et al., 2023; Ibrahim et al., 2023; Kumeroa et al., 2022; Stenerson & Halpenny, 2018). However, five terpenes, namely β-cis-caryophyllene, α-Zingiberene, Thymoquinone, α-Citral, and β-Citronellene, were not detected in the documented referenced publications. While the identified terpenes aligned with established major terpenes in Cannabis Sativa L., the non-detection of these specific compounds in the literature suggests potential variations or unique characteristics in the hemp samples analysed or the techniques used. This discrepancy could stem from differences in hemp varieties, cultivation conditions, or analytical techniques employed in the present study compared to those used in the cited publications. In our study, we employed the Headspace Solid-Phase Microextraction – Gas Chromatography-Mass Spectrometry (HS-SPME – GC-MS) method, successfully identifying and quantifying 29 terpenes. Comparatively, Giovannoni et al. (2023) quantified 20 terpenes using Solid Phase Microextraction combined with Gas Chromatography and Flame Ionization Detection (SPME/GC-FID). Ibrahim et al. (2023) utilized Gas Chromatography-Flame Ionization Detector (GC-FID) for the identification and validation of 10 major terpenes. Our study, employing HS-SPME – GC-MS, identified 29 terpenes, surpassing the numbers reported in the referenced articles.,

which can be attributed to the meticulous optimization of our HS-SPME method, ensuring enhanced sensitivity and a broader scope for terpene detection. Furthermore, Kumeroa et al. (2022) employed dynamic headspace sampling and Gas Chromatography coupled to Mass Spectrometry (GC-MS) to tentatively identify 35 volatile compounds. Stenerson & Halpenny (2018) utilized Headspace Solid-Phase Microextraction (HS-SPME) and GC–Mass Spectrometry (MS) to identify 42 terpenes. While Kumeroa and Stenerson's techniques, which mirror ours, potentially suggest that certain parameters within these techniques might impact the results, parameters such as extraction time, temperature, and fibre coating can influence the efficiency of HS-SPME in capturing volatile compounds. Even though the techniques are similar, subtle differences in these parameters might lead to variations in the number and types of terpenes detected. The absence of these terpenes in referenced publications may also prompt further exploration into the chemical diversity of hemp terpenes, in addition to possibly delving into the influence of specific parameters on the HS-SPME technique, which could provide insights into the conditions fostering the detection of previously unnoticed terpenes. The volatile compounds β-pinene, β-myrcene, α-pinene, limonene, and α-caryophyllene identified in the analysis are noteworthy, as they align with the major terpenes commonly found in *Cannabis Sativa* L., as reported in four scientific documents. This alignment underscores the consistency of the identified terpenes with the established terpene profile of *Cannabis Sativa* L. According to Ibrahim et al. (2022), these compounds, along with Linalool, β-caryophyllene (documented in three scientific publications), and α-terpineol (reported in one publication), constitute the top ten major terpenes in hemp. Intriguingly, the analysis did not detect terpinolene and caryophyllene oxide, which are among the top ten terpenes in hemp according to the documented literature. While our optimized technique allowed for the detection of a higher number of terpenes, the nuanced differences in hemp chemistry across studies may influence the presence or absence of specific compounds, highlighting the need for further exploration and refinement in understanding terpene expression in *Cannabis Sativa* L.

### 4.2.2 Quantitative Evaluation of Aromatic Compounds in Savory: Analysing Concentration via Literature References

The analysis of savory's aromatic profile revealed the presence of 29 total terpenes. However, only 18 of these terpenes showed agreement with the previously documented aromatic profile of savory *(Satureja hortensis L.)*, as referenced in four publications (reference “a,b,c,d”, Table 15) (Baser et al., 2004; Jerković et al., 2015; Rodríguez-Solana et al., 2014, 2015).

In hemp, 82% of the identified terpenes matched with those reported in existing scientific publications, while in savory, only 64% of the identified terpenes matched in the literature. This

discrepancy may be attributed to varying factors, primarily the availability of bibliographic material for comparison. The lower correspondence for savory, at 64%, suggests that there might be fewer published studies or less comprehensive data on the terpene profile of savory, contributing to a reduced ability to match identified compounds. The comparative analysis of existing literature failed to identify certain terpenes, namely β-Citronellene, δ-3-carene, δ-sylvestrene, terpinen-4-ol, α-Zingiberene, cis, trans-α-Farnesene, trans-α-Bergamotene, α-Caryophyllene, Thymoquinone, and α-Citral, in both hemp and savory plants. This absence suggests that these specific terpenes may not be prevalent or may exhibit variations in their presence across different plant specimens. Linalool, a monoterpene, was consistently identified in all 4 publications, albeit in minimal quantities (0.1%) in cultivated varieties (KHC Baser et al., 2004). In addition to that, terpenes consistently mentioned across three publications include β-pinene, β-myrcene, limonene, α-Phellandrene, cis-Sabinene hydrate, and α-terpineol. The absence of terpenes such as carvacrol, p-cymene, and γ-terpinene in the analysed samples was notable, especially considering their high percentages in savory oil according to previous research (KHC Baser et al., 2004). This discrepancy underscores the need for more in-depth analyses and further investigation into the specific conditions or factors influencing the terpene composition of the savory samples in this study. Possible explanations could include variations in cultivation practices, environmental factors (early balsamic time), or genetic differences among savory varieties, in addition to the use of different analytical techniques. Addressing these aspects would contribute to a comprehensive understanding of the terpene profile variations and enhance the reliability and applicability of the findings. The comparison of terpene abundances reveals a striking disparity between the plant sources (hemp and savory) and the dietary samples.

Almost all terpenes exhibit significantly higher concentrations in hemp and savory, as indicated by the quantitative data in Table 15. This pronounced difference underscores the distinct and characteristic terpene profiles associated with each plant source. The relatively low levels of terpenes observed in the diet may be attributed to several factors. Firstly, the diet represents a composite mixture of various raw materials, and the terpene content of each individual component may vary. Additionally, the inclusion of diverse ingredients in the diet, which might not be rich sources of terpenes, could dilute the overall terpene concentration. The complex nature of the diet, with a multitude of components, may contribute to the observed lower terpene levels compared to the more concentrated and specific terpene profiles of hemp and savory. Further research and in-depth analyses are necessary to elucidate the specific origins and transformations of terpenes within the dietary composition.

### 4.2.3 Comparative Analysis of Terpene Content: Contrasting Aromatic Profiles of Hemp, Savory and the diet.

The examination of the aromatic profile of hemp reveals nuanced insights into the least abundant terpenes. Notably, the identified compounds surpassed one million in concentration in hemp (α-Thujene, β-pinene, β-myrcene, β-Citronellene, α-pinene, δ-3-carene, δ-sylvestrene, α-Phellandrene, cis-b-ocimene, α-Zingiberene, α-Caryophyllene, Caryophyllene, and cis, trans α-Farnesene), while only five compounds surpassed one million in savory (α-Thujene, β-myrcene, o-cymene, cis-β-ocimene, and Thymoquinone) where the o-cymene represents about the 97.39% w/w of all the identified terpenes, indicating its predominant presence and significant contribution to the overall aromatic composition. Moreover, Terpinen-4-ol, Linalool, α-Ylangene, α-terpineol, Camphor, and Thymoquinone were identified as the least prevalent compounds, showcasing concentrations of 4,704, 3,531, 24,240, 1,987, 6,637, and 2,071, respectively. In contrast, savory exhibits a divergent pattern, with cis-Sabinene hydrate, a-Ylangene, trans-a-Bergamotene, and b-Farnesene emerging as the least representative compounds, quantified at 102, 14,828, 11,040, and 2,973, respectively. These findings emphasize the selectivity of certain terpenes in contributing to the characteristic aromatic profiles of hemp and savory. The discernible discrepancy in terpene abundance between the "diet" item and the respective plant sources, hemp and savory, was expected and can be attributed to the inherent variations in the dietary composition. Generally, dairy cow diets are composed of a mixture of different ingredients. The ingredients used in intensive dairy farms have lower terpene concentrations compared to pastures, which are rich in specific officinal/medicinal plants. In this context, the particularly elevated levels of Linalool in the diet (360,340) compared to hemp (3,531) and savory (92,563) may be influenced by specific dietary components that contribute disproportionately to this monoterpene. Similarly, the substantial increase in α-Terpineol (27,227) and cis-Sabinene hydrate (11,729) in the diet, compared to their respective plant sources, hemp (1,987) and savory (102), could stem from the incorporation of specific feed ingredients rich in these terpenes. The inclusion of alfalfa (Medicago sativa L.) in the unifeed composition hints at the presence of linalool, a monoterpene, in alfalfa, contributing to the observed high levels of linalool in the diet. Alfalfa is a perennial forage crop commonly used in animal diets (Tava & Pecetti, 1997). However, the challenge of establishing correlations for other terpenes in the ration emphasizes the intricate nature of terpene sourcing from diverse raw materials. The unavailability of detailed bibliographic data underscores the complexity of terpene origins in animal feed ingredients. Similarly, the increased levels of α-Terpineol and cis-Sabinene hydrate in the diet, compared to hemp and savory, suggest unique raw material contributions. The limited bibliographic information necessitates additional research to comprehensively understand the terpene origins in animal diets.

As for the compounds represented in Table 16, the volatile compound phenylacetaldehyde, with a substantial presence of 347,861, was notably identified in the diet, as indicated. Its abundance can be attributed to its content within sunflower seeds, as supported by the findings of Guo et al. (2019). Sunflower seeds are recognized for their diverse chemical composition, and the release of phenylacetaldehyde contributes to the overall aromatic profile of the diet. In contrast, the concentrations of phenylacetaldehyde in hemp (119,567) and savory (2,877,823) exhibit distinct levels, suggesting variations in terpene profiles among these sources. Moreover, phenylacetaldehyde stands out as an exception, presenting a concentration in savory, which was eight times higher than in the diet. This significant disparity aligns with findings by Jerković et al., 2014, recognizing Phenylacetaldehyde as a key volatile compound contributing to the distinctive aromatic profile of savory. Vinyl amyl carbinol, identified by Giovannoni et al. (2022), exhibits minimal presence in hemp, while in savory, it stands out as one of the most important alcohols. This distinction is supported by multiple publications, including Rodríguez-Solana et al. (2014), Rodríguez-Solana-Salgado et al. (2014), and KHC Baser et al. (2004), consistently reporting its prevalence in the aromatic profile of savory. The specificity of Vinyl amyl carbinol to savory suggests a unique biosynthetic pathway or genetic regulation in this aromatic plant, contributing to its characteristic aroma. The minimal detection in hemp aligns with the notion that terpene and aroma profiles vary across plant species due to genetic and environmental influences.

*Table 15. Volatile Aroma Compounds in Foods - Terpenes Identified in Hemp, Savory, and Diet*

| TERPENES | Diet | Hemp | Savory | Ref |
|---|---|---|---|---|
| **α-Thujene** | 2,237 | 14,986,464 | 1,519,245 | b,g,h |
| **β-pinene** | 6,229 | 4,145,412 | 228,099 | b,c,d,e,f,g,h |
| **β-myrcene** | 24,101 | 4,392,393 | 2,963,259 | b,c,d,e,f,g,h |
| **β-Citronellene** | 1,952 | 1,218,663 | 46,329 | - |
| **Camphene** | 7,154 | 140,256 | 109,105 | b,d,g,h |
| **α-pinene** | 10,156 | 5,718,351 | 266,944 | b,d,e,f,g,h |
| **δ-3-carene** | 7,653 | 1,307,874 | 304,768 | e,g,h |
| **o-cymene** | 32,775 | 279,724 | 48,472,745 | c,g,h |
| **Limonene** | 121,997 | 661,797 | 305,389 | b,c,d,e,f,g,h |
| **δ-sylvestrene** | 130,886 | 1,297,677 | 765,794 | e |
| **T-β-ocimene** | 772 | 272,294 | 766,040 | d,e,g |
| **Terpinen-4-ol** | 3,261 | 4,704 | 141,027 | h |
| **α-Phellandrene** | 4,580 | 1,308,051 | 305,118 | b,c,d,e,g |
| **C-Sabinenehydrate** | 11,729 | 118,365 | 102 | b,c,d,g |
| **Linalool** | 360,340 | 3,531 | 92,563 | a,b,c,d,f,g,h |
| **C-β-ocimene** | 9,095 | 1,421,951 | 5,627,103 | b,e,g |
| **α-Ylangene** | 4,917 | 24,240 | 14,828 | c,g,h |
| **β-ciscaryophyllene** | 645 | 3,493,014 | 397,194 | d |
| **α-Zingiberene** | 3,936 | 1,520,828 | 174,690 | - |
| **C,T-α-Farnesene** | 6,590 | 4,133,683 | 469,370 | e,g |
| **Caryophyllene** | 5,631 | 3,490,101 | 392,595 | b,c,f,g,h |
| **T-α-Bergamotene** | 63 | 475,704 | 11,040 | h |
| **α-caryophyllene** | 62 | 2,664,489 | 77,642 | e,f,g,h |
| **β-Farnesene** | 79 | 488,009 | 2,973 | c,e,g,h |
| **α-terpineol** | 27,277 | 1,987 | 46,677 | a,c,d,h |
| **Camphor** | 2,379 | 6,637 | 40,367 | b,c,e |
| **Thymoquinone** | 195 | 2,071 | 1,790,016 | - |
| **α-Citral** | 30,067 | - | 141,099 | - |
| **Caryophylladienol II** | 645 | 87,600 | 13,825 | - |
| **Total** | 664,150 | 58,977,713 | 74,140,259 | |

Reference Savory: a: (Jerković et al., 2015), b: (Rodríguez-Solana et al., 2014), c: (Rodríguez-Solana et al., 2015), d: (Baser et al., 2004). Reference Hemp: e: (Kumeroa et al., 2022), f: (Ibrahim et al., 2023), g: (Giovannoni et al., 2023), h: (Stenerson & Halpenny, 2018)

*Table 16. Volatile Aroma Compounds in Foods – Other Compounds Identified in Hemp, Savory, and Diet*

| Common Name | Diet | Hemp | Savory | Ref |
|---|---|---|---|---|
| **Vinyl amyl carbinol** | 641,434 | 102,269 | 1,389,164 | d |
| **Phenylacetaldehyde** | 347,861 | 119,567 | 2,877,823 | a |

d: (Baser et al., 2004), a: (Jerković et al., 2015)

## 4.3. Exploring the Terpene Dynamics: Aromatic Profile of Milk Under Different Dietary Treatments

Table 17 presents an analysis of VOC in the milk of cows subjected to a basal diet and diets with supplementation with hemp or savory, in addition to analysis of cow and period effects. A review of the available literature revealed a notable absence of studies employing the same milk analysis method (HS-SPME-GC-SM procedure) for characterizing volatile aromatic compounds in both hemp and savory. Among the compounds identified, the ones that exhibited statistically significant differences ($P$<0.10) in the milk of cows across the three experimental groups of cows encompass fourteen terpenes, and one aldehyde. Upon comprehensive examination of Table 17, encompassing all compound classes, no statistically significant differences associated with the experimental period were discerned ($P$<0.10). In essence, the aromatic profile of the milk exhibited relative stability throughout the experimental period, indicating a lack of discernible alterations attributable to potential fluctuations in the composition of the basal diet. Individual cows exhibited distinct aromatic profiles in multiple instances, manifesting tendential and significant effects (Table 17). This underscores the noteworthy influence of individual animals in shaping the transfer of aromatic molecules from dietary intake to the resultant milk product. Such variations among individuals can be attributed to factors encompassing feeding behaviour, ruminal and intestinal digestive processes, as well as the intricacies of transformation and metabolic transfer mechanisms. The experimental treatment, as in the administration of hemp and savory to cows had statistically significant effects for some terpene compounds including: δ -3-carene, o-cymene, Limonene, δ-sylvestrene, cis-β-ocimene, α-terpineol, a-Zingiberene, cis-trans-α-Farnesene, Caryophyllene, trans-α-Bergamotene, and α-Caryophyllene ($P$<0.10).

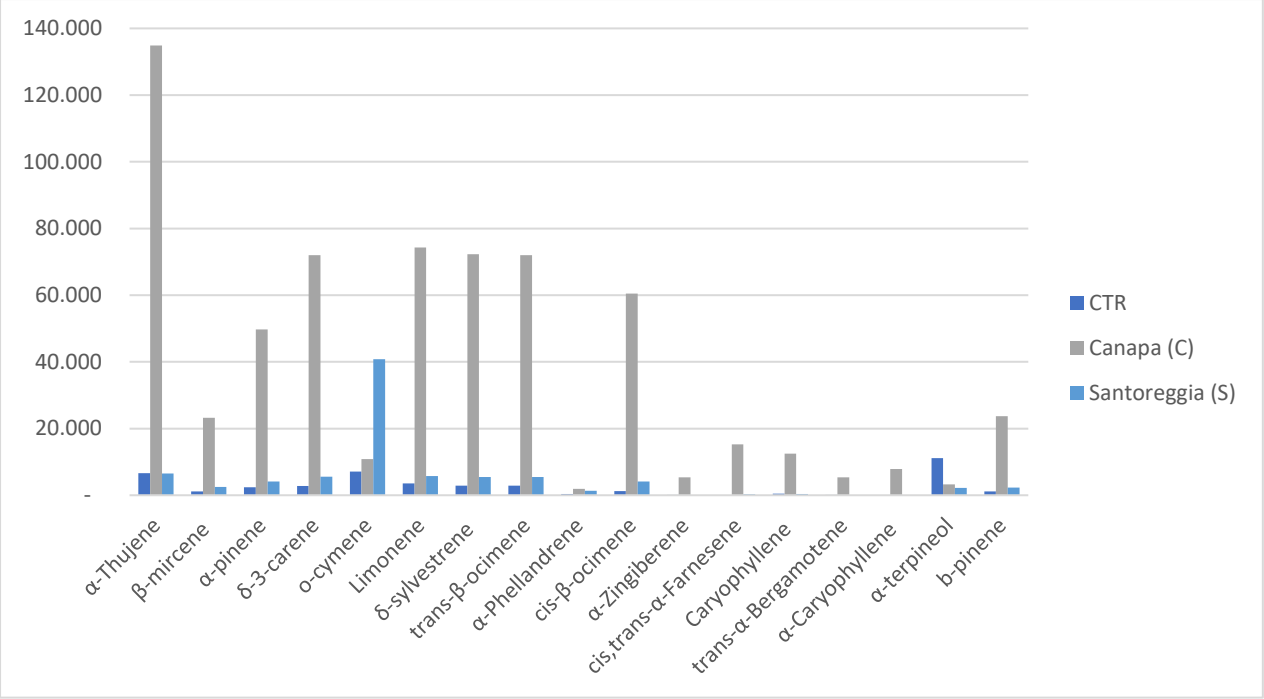



*Figure 9. Effect of diet treatments on the terpene profile in milk*

Illustrated in Figure 9, the introduction of hemp into the diet exerted a notably more pronounced impact compared to savory in augmenting the milk with terpenes. The milk composition seemingly reflects the aromatic profile of hemp *(Cannabis sativa L.).* Notably, among the statistically significant terpenes, β-pinene, β-myrcene, α-pinene, Limonene, and α-Caryophyllene were observed, all of which constitute integral components of the ten major terpenes identified in *Cannabis sativa L.* (Ibrahim et al., 2022). For the milk obtained by feeding cows with savory (S), much more limited terpene concentration peaks were observed (Graph 1) except in the case of o-cymene. The addition of hemp to the diet increased the concentration of the following terpenes in milk: α -Thujene, β -pinene, β -Myrcene, α -pinene, δ -3-carene, Limonene, δ - sylvestrene, trans- β -ocimene, cis- β -ocimene, α -Zingiberene, cistrans-α-Farnesene, Caryophyllene, trans-α-Bergamotene, α -Caryophyllene ($P<0.10$). In the aldehyde family, Phenylacetaldehyde increases following administration of hemp to cows (2,177 (CTR) and 25,150 (C), respectively). The only compounds that have been reduced however were α -terpineol and Vinyl amyl carbinol. The reduction in α-terpineol and Vinyl amyl carbinol concentrations was unexpected, as all three groups of cows received the same basal diet representing 100% of the total intake allowance (TIA) for the control (CTR) group, and 98% for both the hemp (H) and savory (S) groups. Therefore, it was anticipated that the milk of all three groups of cows would exhibit similar content of α-terpineol and Vinyl amyl carbinol. The unexpected decrease in the concentrations of these compounds suggests potential factors influencing their transfer from the diet to milk, warranting further investigation

into the underlying mechanisms. Indeed, α-terpineol was the sole terpene found in higher quantities in the diet compared to hemp. The observed lower levels of α-terpineol and Vinyl amyl carbinol in hemp and savory, relative to the diet, may be attributed to various factors. One potential explanation could be the dilution effect, where the concentration of certain compounds in milk may diminish due to the volume of feed consumed. This could result in reduced observed concentrations in plant samples. Secondly, during the analytical process, particularly in techniques like gas chromatography-mass spectrometry (GC-MS), compounds within the sample matrix may compete for detection. This competition arises during the separation and ionization stages of GC-MS analysis, where compounds with similar chemical properties or structures can interfere with the detection of target compounds. The addition of savory to the diet resulted in an increase in the concentration of o-cymene in milk and, similarly to hemp, reduced a-terpineol in accordance with the different content of these terpenes in the diet. Conversely, the concentration of Vinyl amyl carbinol was higher in the milk of cows taking the basic diet compared to cows fed hemp and savory. In fact, savory provides a significantly lower quantity of terpenes than hemp and therefore the "competitive" effect was probably milder. When interpreting the values, it is necessary to take into account that the quantity of diet ingested by the animals was significantly higher than the two herbs covered by this research. In fact, hemp and savory constituted just over 2% of the dry matter ingested by the animals. For example, for hemp, α-terpineol had high concentrations because these terpenes were also present in significant quantities in the basic diet. Additionally, Lejonklev et al. (2013) conducted a related study investigating the transfer of volatile terpenes from essential oils, specifically caraway seed and oregano plant essential oils, into cow's milk through respiratory and gastrointestinal exposure (J. Lejonklev et al., 2013). Their findings align with our observations, indicating a rapid and immediate effect on milk composition after exposure to terpene-rich sources, supporting the potential for manipulating milk composition through dietary means. This study emphasizes the importance of considering the composition of the animal's diet in interpreting terpene profiles in milk.

*Table 17. Aromatic Analysis of Milk: Compounds Identified in Milk Through HS-SPME-GC-MS*

| Common Name | Experimental Treatment (TRT) | | | SE[4] | P-Value | | | | Contrasts[9] | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | Control[1] | Hemp[2] (H) | Savory[3] (S) | | TRT[5] | Period[6] | Group[7] | Cow[8] | CTRL vs. H | CTRL vs. S | H vs. S |
| **TERPENES** | | | | | | | | | | | |
| α -Thujene | 6,588 | 134,911 | 6,508 | 27,245 | 0.12 | 0.52 | 0.51 | 0.07 | **0.08** | 1.00 | **0.08** |
| β -pinene | 1,132 | 23,702 | 2,256 | 4,745 | 0.12 | 0.56 | 0.50 | 0.10 | **0.08** | 0.88 | **0.09** |
| β -myrcene | 1,174 | 23,220 | 2,490 | 4,850 | 0.13 | 0.57 | 0.51 | 0.11 | **0.08** | 0.87 | **0.09** |
| β -Citronellene | 965 | 5,436 | 2,256 | 1,158 | 0.20 | 0.46 | 0.52 | 0.74 | 0.11 | 0.51 | 0.19 |
| Camphene | 10,245 | 8,261 | 7,712 | 692 | 0.21 | 0.15 | 0.24 | 0.11 | 0.18 | 0.12 | 0.63 |
| α -pinene | 2,353 | 49,737 | 4,094 | 10,213 | 0.13 | 0.53 | 0.52 | 0.09 | **0.08** | 0.92 | **0.09** |
| δ -3-carene | 2,752 | 72,007 | 5,592 | 14,098 | 0.11 | 0.47 | 0.53 | **0.04** | **0.07** | 0.90 | **0.08** |
| o-cymene | 7,086 | 10,834 | 40,765 | 5,125 | 0.07 | 0.52 | 0.62 | **0.01** | 0.66 | **0.04** | **0.05** |
| Limonene | 3,511 | 74,281 | 5,769 | 14,479 | 0.11 | 0.48 | 0.56 | **0.04** | **0.07** | 0.92 | **0.08** |
| δ -sylvestrene | 2,865 | 72,249 | 5,506 | 14,173 | 0.11 | 0.47 | 0.55 | **0.04** | **0.07** | 0.91 | **0.08** |
| Tb-ocimene | 2,865 | 71,953 | 5,506 | 14,211 | 0.12 | 0.48 | 0.56 | **0.04** | **0.08** | 0.91 | **0.08** |
| terpinen-4-ol | 1,840 | 1,724 | 1,899 | 193 | 0.83 | 0.46 | 0.66 | **0.04** | 0.71 | 0.85 | 0.59 |
| α -Phellandrene | 368 | 1,950 | 1,357 | 408 | 0.21 | 0.32 | 0.37 | **0.05** | 0.11 | 0.23 | 0.41 |
| cis-Sabinene hydrate | 33 | 317 | 117 | 76 | 0.21 | 0.31 | 0.45 | **0.01** | 0.12 | 0.51 | 0.20 |
| Linalool | 2,407 | 2,542 | 1,696 | 490 | 0.54 | 0.43 | 0.91 | 0.68 | 0.86 | 0.41 | 0.35 |
| C- β -ocimene | 1,255 | 60,491 | 4.125 | 11,968 | 0.11 | 0.48 | 0.56 | 0.05 | **0.07** | 0.88 | 0.08 |
| α -Ylangene | 1,356 | 1,051 | 1,231 | 191 | 0.61 | 0.18 | 0.41 | 0.70 | 0.38 | 0.69 | 0.58 |
| β -cis-caryophyllene | 6,842 | 12,572 | 6,066 | 1,652 | 0.18 | 0.82 | 0.32 | 0.27 | 0.13 | 0.77 | 0.11 |
| a-Zingiberene | 23 | 5,371 | 0 | 713 | 0.05 | 0.67 | 0.66 | 0.15 | **0.03** | 0.98 | **0.03** |
| C,T-α-Farnesene | - | 15,256 | 283 | 1,909 | 0.05 | 0.67 | 0.70 | 0.12 | **0.03** | 0.93 | **0.03** |
| Caryophyllene | 430 | 12,513 | 364 | 1,565 | 0.05 | 0.69 | 0.69 | 0.16 | **0.03** | 0.98 | **0.03** |
| T-α-Bergamotene | 23 | 5,371 | 0 | 713 | 0.05 | 0.67 | 0.66 | 0.15 | **0.03** | 0.98 | **0.03** |
| α -Caryophyllene | 0 | 7,851 | 0 | 996 | 0.05 | 0.71 | 0.71 | 0.12 | **0.03** | 1.00 | **0.03** |
| b-Farnesene | 28 | 3,301 | 48 | 1,194 | 0.29 | 0.60 | 0.60 | 0.32 | 0.19 | 0.99 | 0.19 |
| α -terpineol | 11,114 | 3,267 | 2,166 | 1,579 | 0.09 | 0.79 | 0.72 | 0.50 | **0.07** | **0.06** | 0.67 |
| Camphor | 343 | 354 | 478 | 143 | 0.78 | 0.17 | 0.42 | 0.40 | 0.96 | 0.57 | 0.60 |
| Thymoquinone | 879 | 639 | 827 | 119 | 0.47 | 0.10 | 0.83 | 0.75 | 0.29 | 0.79 | 0.38 |
| α -Citral | 348 | 120 | 208 | 127 | 0.55 | 0.41 | 0.54 | 0.69 | 0.33 | 0.51 | 0.67 |
| Caryophylladienol II | 0 | 4,947 | 0 | 764 | 0.07 | 0.50 | 0.50 | 0.08 | **0.04** | 1.00 | **0.04** |
| **Other Compounds*** | | | | | | | | | | | |
| Vinyl amyl carbinol | 90,600 | 38,164 | 33,608 | 13,412 | 0.15 | 0.58 | 0.69 | 0.49 | 0.11 | **0.10** | 0.83 |
| Phenylacetaldehyde | 2,177 | 25,150 | 10,206 | 4,543 | 0.13 | 0.51 | 0.44 | **0.01** | **0.07** | 0.34 | 0.15 |

[1]CTR: Control Treatment (Unifeed Only), [2]H: Hemp Treatment (Hemp + Unifeed), [3]S: Savory Treatment (Savory + Unifeed), [4]SE: Standard Error, [5]TRT: Experimental Treatment Effect, [6]Period: Effect of the Experimental Period, [7]Group: Cow Group Effect (2 for each group), [8]Cow: Cow Effect Within the Group, [9]Contrasts: Comparison Between Different Treatment Groups, *: Other Compounds Included 1 alcohol and 1 Aldehyde

## 4.4 Exploring the Fatty Acids Dynamics: Aromatic Profile of Milk Under Different Dietary Treatments

Table 18 and Table 19 present the milk FA profile of cows fed with the three experimental diets (TRT): conventional diet (CTR) and diets supplemented with hemp (H) or savory (S). The GC×GC analysis revealed the presence in milk of 86 compounds corresponding to 28 SFA, 37 MUFA, 21 PUFA. For reasons of space, the FA partially identified were summed in groups.

### 4.4.1 Experimental period, group, and cow effects

In various instances, individual cows displayed unique FAs profiles, demonstrating both tendential and significant effects (see Table 18 and 19). Particularly, the presence of SFA notably the even SFA (C14:0 and C16:0) showed statistically significant differences between individual cows *(P<0.05)*. Variations between individual cows were also significant for odd SFA (C15:0 and C17:0) *(P<0.05)*. Variations were also observed for the group of MUFA C18-C24 with a statistically significant *P<0.05*. The PUFA showed also statistical significance among individual cows. This highlights the considerable impact of individual animals in determining the presence of FAs in the resulting milk product, with variations attributed to factors such as the rumen fermentations and the complex metabolic and udder biosynthesis and transformations mechanisms but also other secondary aspects like feeding behaviour, and intestinal digestive processes.

### 4.4.2 Diet effects

Also, about the diet treatment (TRT), and considering all compound categories, while the data collected presented various trends and observations, no definitive outcomes reached a level of statistical confidence. The analysis of even SFA, such as Butyric acid (C4:0) to Behenic acid (C22:0), revealed no significant variations among the control, hemp, and savory treatments. For instance, Palmitic acid (C16:0), the predominant even SFA, exhibited comparable levels across all treatments (28.51%, 28.29%, and 29.02% for CTR, H, and S diet, respectively).

The examination of odd SFA (Pentanoic acid (C5:0) to Heneicosanoic acid (C21:0)) indicated minimal variations among treatments. While some individual odd-chain SFAs showed slight differences, the overall concentrations remained consistent across Control, Hemp, and Savory. Notably, Pentadecanoic acid (C15:0) and Heptadecanoic acid (C17:0) demonstrated stable levels across treatments. The analysis of branched-chain SFAs (Iso-Tridecanoic acid (C13:0 iso) to Anteiso-Nonadecanoic acid (C19:0 anteiso)) showed no substantial differences among treatments. The concentrations of these SFAs were consistent across the control, hemp, and savory diets. For example, C15:0 iso and C17:0 anteiso exhibited comparable levels.

Furthermore, the analysis of MUFA in the C10-C17 and in the C18-C24 ranges showed no significant differences among the control, hemp, and savory treatments. Individual fatty acids within this category, such as cis-10-Heptadecenoic acid (C10:1*c*9) and Oleic acid (C18:1*c*9), the predominant MUFA, exhibited consistent levels. In addition to that, the concentrations of PUFA remained stable across control, hemp, and savory diets.

### 4.4.3 Diet effect on desaturase indices

The desaturase indices, including C10:1, C14:1, C16:1, and C18:1, exhibited consistent levels across all treatments. These indices provide insights into the activity of desaturase enzymes involved in fatty acid metabolism. Illustrated in Graph 2, the introduction of hemp or savory into the diet of the cows did not exert a pronounced impact compared to the conventional diet in augmenting the milk with SFAs. When comparing the FAs profile of cows that had a different diet, it can be noticed that the amount of SFAs barely changed. For instance, a slight unsignificant augmentation for the SFA C10:0, can be seen (P-value = 0,397). Depicted in Graph 3, incorporating hemp or savory into the cows' diet did not have a substantial effect compared to the standard diet in enhancing the milk with MUFAs or PUFAs. Upon assessing the fatty acid profiles of cows under different dietary conditions, the analysis revealed consistent levels of polyunsaturated and monounsaturated fatty acids. The incorporation of hemp or savory into the diet did not induce significant changes in the composition of saturated, monounsaturated, or polyunsaturated fatty acids in milk.

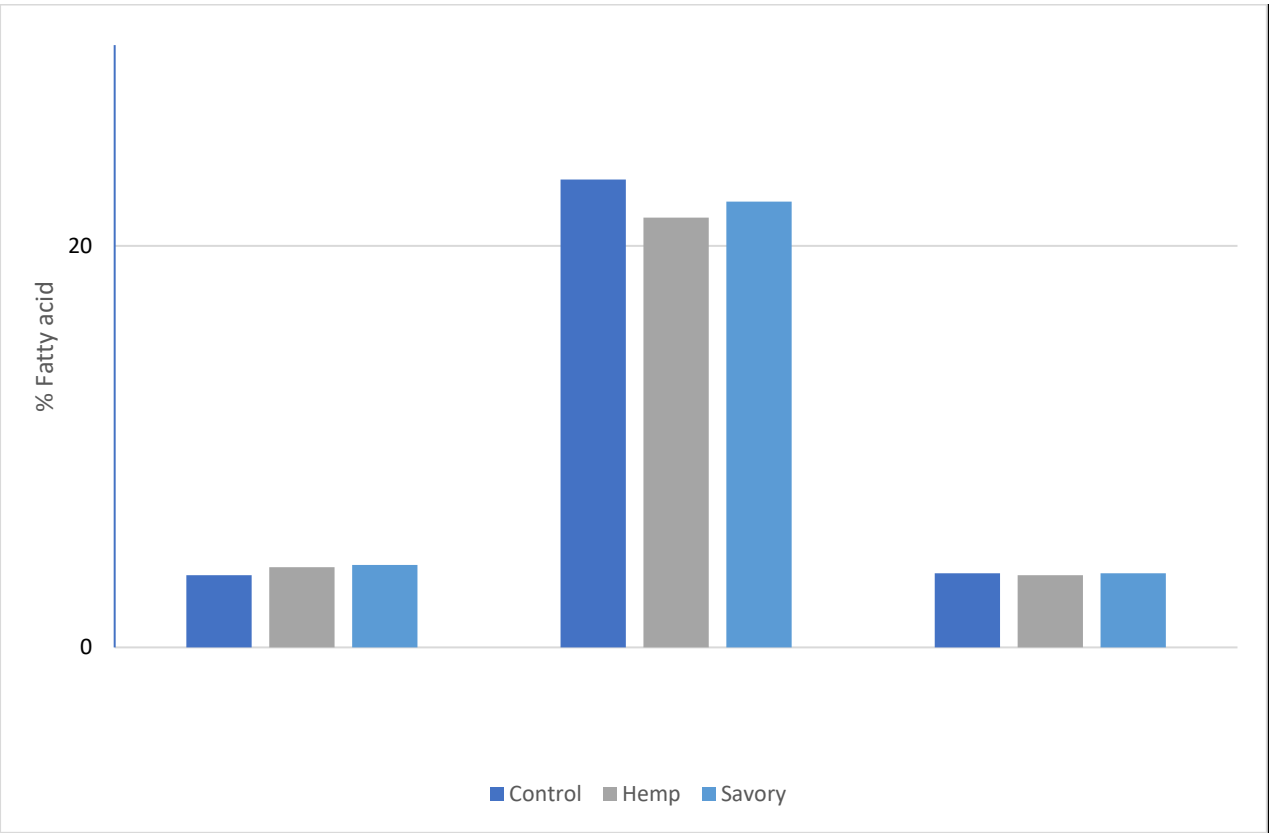



*Figure 10. Effect of diet on the SFA profile in milk*

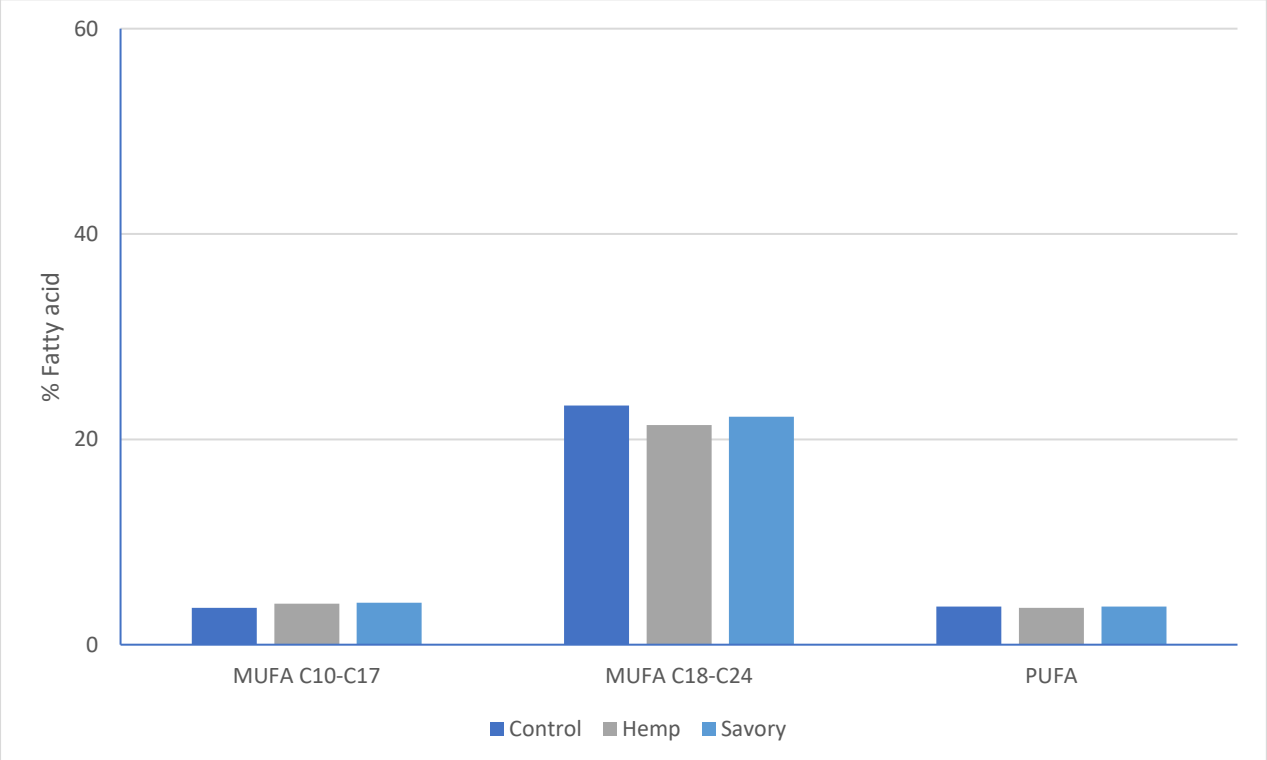



*Figure 11. Effect of diet on the MUFA and PUFA profile in milk*

*Table 18. Fatty Acid Profile Analysis of Milk: Saturated Fatty Acids Identified in Milk Through GC×GC*

| Item | Experimental Treatment (TRT) | | | SE[4] | P-Value | | | | Contrasts[9] | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | Control[1] | Hemp[2] | Savory[3] | | TRT[5] | Period[6] | Group[7] | Cow[8] | CTR vs. H | CTR vs. S | H vs. S |
| **SFA[10]** | 69.20 | 70.70 | 69.70 | 1.170 | 0.56 | 0.10 | 0.41 | **0.01** | 0.342 | 0.722 | 0.504 |
| **Even SFA[11]** | 65.20 | 66.40 | 65.40 | 1.225 | 0.62 | 0.09 | 0.50 | **0.01** | 0.413 | 0.888 | 0.482 |
| C4:0 | 3.15 | 3.19 | 3.12 | 0.313 | 0.99 | 0.26 | 0.72 | 0.38 | 0.925 | 0.890 | 0.964 |
| C6:0 | 2.92 | 2.89 | 2.74 | 0.246 | 0.87 | 0.21 | 0.80 | 0.64 | 0.936 | 0.708 | 0.654 |
| C8:0 | 1.99 | 1.98 | 1.85 | 0.158 | 0.80 | 0.20 | 0.93 | 0.74 | 0.976 | 0.612 | 0.593 |
| C10:0 | 3.99 | 4.44 | 4.04 | 0.295 | 0.59 | 0.20 | 0.99 | 0.24 | 0.397 | 0.441 | 0.918 |
| C12:0 | 4.09 | 4.97 | 4.41 | 0.321 | 0.34 | 0.23 | 0.84 | 0.23 | 0.193 | 0.341 | 0.559 |
| C14:0 | 11.68 | 12.42 | 12.11 | 0.339 | 0.45 | 0.15 | 0.22 | **0.01** | 0.261 | 0.579 | 0.465 |
| C16:0 | 28.51 | 28.29 | 29.02 | 0.469 | 0.61 | 0.25 | 0.18 | **0.02** | 0.767 | 0.384 | 0.523 |
| C18:0 | 8.71 | 8.06 | 7.96 | 0.733 | 0.76 | 0.85 | 0.48 | 0.89 | 0595 | 0.930 | 0.543 |
| C20:0 | 0.11 | 0.16 | 0.11 | 0.038 | 0.62 | 0.86 | 0.47 | 0.43 | 0.464 | 0.420 | 0.923 |
| C22:0 | 0.01 | 0.02 | 0.01 | 0.017 | 0.91 | 0.83 | 0.83 | 0.72 | 0.716 | 0.773 | 0.937 |
| **Odd SFA[12]** | 2.40 | 2.60 | 2.70 | 0.344 | 0.65 | 0.59 | 0.42 | 0.07 | 0.512 | 0.423 | 0.855 |
| C5:0 | 0.2 | 0.14 | 0.21 | 0.058 | 0.70 | 0.47 | 0.82 | 0.39 | 0.537 | 0.488 | 0.927 |
| C7:0 | 0.10 | 0.12 | 0.11 | 0.020 | 0.74 | 0.68 | 0.71 | 0.79 | 0.515 | 0.900 | 0.587 |
| C9:0 | 0.08 | 0.10 | 0.10 | 0.020 | 0.70 | 0.74 | 0.62 | 0.68 | 0.533 | 0.941 | 0.493 |
| C11:0 | 0.12 | 0.16 | 0.17 | 0.037 | 0.63 | 0.77 | 0.62 | 0.70 | 0.458 | 0.963 | 0.436 |
| C13:0 | 0.12 | 0.18 | 0.17 | 0.032 | 0.54 | 0.79 | 0.41 | 0.13 | 0.335 | 0.782 | 0.446 |
| C15:0 | 1.14 | 1.39 | 1.33 | 0.135 | 0.52 | 0.58 | 0.26 | **0.05** | 0.327 | 0.800 | 0.423 |
| C17:0 | 0.57 | 0.44 | 0.57 | 0.049 | 0.30 | 0.15 | 0.85 | **0.02** | 0.205 | 0.202 | 0.988 |
| C19:0 | 0.04 | 0.08 | 0.04 | 0.042 | 0.73 | 0.29 | 0.80 | 0.45 | 0.501 | 0.573 | 0.897 |
| C21:0 | 0.02 | 0.03 | 0.01 | 0.015 | 0.66 | 0.30 | 0.60 | 0.37 | 0.492 | 0.455 | 0.940 |
| **BCSFA[13]** | 1.70 | 1.60 | 1.60 | 0.155 | 0.86 | 0.35 | 0.33 | 0.46 | 0.625 | 0.752 | 0.850 |
| C13:0 *iso* | 0.03 | 0.02 | 0.03 | 0.007 | 0.81 | 0.51 | 0.92 | 0.31 | 0.564 | 0728 | 0.803 |
| C14:0 *iso* | 0.07 | 0.06 | 0.07 | 0.009 | 0.78 | 0.47 | 0.33 | 0.41 | 0.788 | 0.529 | 0.698 |
| C15:0 *iso* | 0.21 | 0.19 | 0.18 | 0.017 | 0.51 | 0.50 | 0.36 | 0.20 | 0.397 | 0.843 | 0.325 |
| C15:0 *anteiso* | 0.36 | 0.44 | 0.41 | 0.024 | 0.30 | 0.29 | 0.56 | **0.01** | 0.165 | 0.468 | 0.336 |
| C16:0 *iso* | 0.20 | 0.16 | 0.19 | 0.034 | 0.76 | 0.46 | 0.63 | 0.42 | 0.519 | 0.637 | 0.843 |
| C17:0 *iso* | 0.29 | 0.22 | 0.28 | 0.038 | 0.46 | 0.31 | 0.44 | **0.02** | 0.292 | 0.356 | 0.842 |
| C17:0 *anteiso* | 0.41 | 0.38 | 0.40 | 0.017 | 0.58 | 0.06 | 0.41 | **0.00** | 0.370 | 0.456 | 0.839 |
| C18:0 *iso* | 0.05 | 0.02 | 0.04 | 0.009 | 0.38 | 0.10 | 0.18 | **0.02** | 0.212 | 0.429 | 0.496 |
| C19:0 *anteiso* | 0.08 | 0.12 | 0.05 | 0.064 | 0.77 | 0.41 | 0.68 | 0.25 | 0.702 | 0.518 | 0.767 |

[1]CTR: Control Treatment (Unifeed Only), [2]H: Hemp Treatment (Hemp + Unifeed), [3]S: Savory Treatment (Savory + Unifeed), [4]SE: Standard Error, [5]TRT: Experimental Treatment Effect, [6]Period: Effect of the Experimental Period, [7]Group: Cow Group Effect (2 for each group), [8]Cow: Cow Effect Within the Group, [9]Contrasts: Comparison Between Different Treatment Groups, [10]SFA: Saturated Fatty Acids, [11]Even SFA: Even Saturated Fatty Acids, [12]Odd SFA: Odd Saturated Fatty Acids, [13]BCSFA: Branched Chain Saturated Fatty Acids

*Table 19. Fatty Acid Profile Analysis of Milk: MUFA and PUFA Identified in Milk Through GC × GC and Desaturase Indices*

| Item | Experimental Treatment (TRT) | | | SE[4] | P-Value | | | | Contrasts[9] | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | Control[1] | Hemp[2] | Savory[3] | | TRT[5] | Period[6] | Group[7] | Cow[8] | CTR vs. H | CTR vs. S | H vs. S |
| **MUFA[10]** | 26.9 | 25.5 | 26.3 | 1.182 | 0.56 | 0.13 | 0.33 | **0.01** | 0.34 | 0.65 | 0.54 |
| **MUFA C10-C17** | 3.6 | 4.0 | 4.1 | 0.609 | 0.76 | 0.59 | 0.71 | 0.67 | 0.58 | 0.55 | 0.96 |
| C10:1 *c*9 | 0.6 | 0.9 | 0.7 | 0.253 | 0.62 | 0.17 | 0.79 | 0.30 | 0.40 | 0.84 | 0.50 |
| ∑C12:1 *cis** | 0.2 | 0.3 | 0.3 | 0.072 | 0.61 | 0.32 | 0.52 | 0.27 | 0.38 | 0.60 | 0.67 |
| C14:1 *c*9 | 0.8 | 0.9 | 0.9 | 0.168 | 0.62 | 0.61 | 0.18 | 0.27 | 0.54 | 0.40 | 0.77 |
| ∑C14:1 others* | 0.01 | 0.01 | 0.01 | 0.008 | 0.89 | 0.28 | 0.09 | 0.29 | 0.88 | 0.79 | 0.68 |
| ∑C15:1* | 0.01 | 0.01 | 0.01 | 0.006 | 0.53 | 0.11 | 0.53 | 0.44 | 0.53 | 0.31 | 0.62 |
| C16:1 *c*9 | 1.3 | 1.3 | 1.4 | 0.130 | 0.55 | 0.32 | 0.31 | 0.16 | 0.77 | 0.46 | 0.34 |
| ∑C16:1 others* | 0.4 | 0.4 | 0.4 | 0.028 | 0.82 | 0.05 | 0.22 | 0.06 | 0.91 | 0.67 | 0.59 |
| ∑C17:1 others* | 0.3 | 0.3 | 0.3 | 0.025 | 0.46 | 0.05 | 0.10 | 0.31 | 0.36 | 0.82 | 0.29 |
| **MUFA C18-C24** | 23.3 | 21.4 | 22.2 | 1.334 | 0.50 | 0.15 | 0.34 | **0.01** | 0.29 | 0.51 | 0.60 |
| C18:1 *c*9 | 20.5 | 18.8 | 19.5 | 1.323 | 0.55 | 0.13 | 0.29 | **0.02** | 0.33 | 0.55 | 0.64 |
| C18:1 *c*11 | 0.3 | 0.4 | 0.7 | 0.253 | 0.49 | 0.82 | 0.46 | 0.62 | 0.83 | 0.31 | 0.38 |
| C18:1 *t*11 | 0.6 | 0.5 | 0.4 | 0.389 | 0.89 | 0.47 | 0.79 | **0.01** | 0.91 | 0.68 | 0.76 |
| C18:1 *c*15 | 0.01 | 0.01 | 0.01 | 0.009 | 0.22 | 0.07 | 0.86 | 0.54 | 0.15 | 0.94 | 0.16 |
| ∑C18:1 others* | 1.4 | 1.2 | 1.2 | 0.326 | 0.86 | 0.34 | 0.55 | **0.01** | 0.66 | 0.69 | 0.97 |
| Total C24:1* | 0.2 | 0.2 | 0.1 | 0.076 | 0.62 | 0.13 | 0.73 | 0.39 | 0.78 | 0.53 | 0.39 |
| **PUFA[11]** | 3.7 | 3.6 | 3.7 | 0.168 | 0.88 | 0.06 | 0.18 | **0.02** | 0.81 | 0.83 | 0.65 |
| ∑C18:2 *t** | 0.8 | 0.8 | 0.8 | 0.058 | 0.53 | 0.05 | 0.15 | **0.02** | 0.34 | 0.42 | 0.83 |
| ∑C18:2 *c** | 2.1 | 2.1 | 2.2 | 0.074 | 0.78 | 0.04 | 0.17 | **0.01** | 0.72 | 0.77 | 0.53 |
| ∑C18:3* | 0.5 | 0.4 | 0.4 | 0.055 | 0.58 | 0.8 | 0.64 | 0.59 | 0.38 | 0.45 | 0.86 |
| ∑C20-C24** | 0.3 | 0.3 | 0.3 | 0.051 | 0.78 | 0.35 | 0.62 | 0.24 | 0.64 | 0.86 | 0.54 |
| **Desaturase Indices*** ** | | | | | | | | | | | |
| C10:1 | 0.1 | 0.2 | 0.1 | 0.011 | 0.61 | 0.16 | 0.56 | 0.41 | 0.38 | 0.73 | 0.55 |
| C14:1 | 0.1 | 0.1 | 0.1 | 0.004 | 0.70 | 0.82 | 0.19 | 0.18 | 0.59 | 0.46 | 0.81 |
| C18:1 | 0.7 | 0.7 | 0.7 | 0.018 | 0.72 | 0.36 | 0.72 | **0.01** | 0.87 | 0.58 | 0.49 |

[1]CTR: Control Treatment (Unifeed Only), [2]H: Hemp Treatment (Hemp + Unifeed), [3]S: Savory Treatment (Savory + Unifeed), [4]SE: Standard Error, [5]TRT: Experimental Treatment Effect, [6]Period: Effect of the Experimental Period, [7]Group: Cow Group Effect (2 for each group), [8]Cow: Cow Effect Within the Group, [9]Contrasts: Comparison Between Different Treatment Groups, [10]MUFA: Monounsaturated Fatty Acids, [11]PUFA: Polyunsaturated Fatty Acids

*: Includes Structural Isomers, **: Includes Omega-3 Fatty Acids, ***: Computed according to (Pegolo et al., 2016b)

## 4.5 Factors contributing to FA profile variations in cow milk

While the data collected presented various trends and observations, the study provides valuable insights into the dynamics of the phenomena under investigation despite the fact that the variations did not reach a level of statistical significance. The FA content among various milk treatments in this study prompts a comprehensive evaluation of several contributing factors. Given the subtle nature of potential variations, ensuring that the chosen techniques can detect nuanced differences becomes paramount. Biological variation in milk composition, potential interaction effects, and temporal fluctuations contribute to the complexity of interpreting fatty acid profiles.

### 4.5.1 Dairy cow digestion and milk composition dynamics

The dynamic nature of the cow's digestive processes and metabolic transformations further adds layers of intricacy to the observed results. Factors such as cow breed and lactation stage, which influence milk composition, need to be considered comprehensively in the context of dietary interventions. Furthermore, acknowledging the specificity of each metabolic pathway and compound class becomes imperative when interpreting results from dietary intervention studies. The nuanced interplay of various factors, including dietary components, microbial activity in the rumen, and enzymatic processes, shapes the fatty acid composition in milk. It is crucial to recognize that alterations in dietary components may not instantaneously manifest in the milk fatty acid profile, as the metabolic transformations within the cow's digestive system involve intricate biochemical processes. Over 95% of the fat in milk consists of triglycerides (TAGs). Milk TAGs are produced through two mechanisms: *de novo* synthesis in mammary epithelial cells and uptake from circulating blood (Tian et al., 2022). Mammary epithelial cells synthesize all short- and medium-chain fatty acids (C4-C14) and approximately half of C16 fatty acids using acetate and β-hydroxybutyrate, both originating from the rumen's fermentation of feed components. Acetate and butyric acid are formed in the rumen, with butyric acid converting to β-hydroxybutyrate during absorption through the rumen epithelium. The remaining half of C16 fatty acids and all long-chain fatty acids (LCFAs) longer than C18 are absorbed from the circulating blood (Lindmark Månsson, 2008). The stability observed in milk fat composition may be linked to the homeostatic mechanisms governing fatty acid metabolism. The mammary gland's ability to synthesize specific fatty acids in response to dietary precursors, as well as the efficient absorption of circulating LCFAs, showcases the regulatory control embedded in the milk fat synthesis process.

### 4.5.2 Contrasts in fatty acid and terpene metabolism in dairy cows

In contrast to the fatty acid profile, the study of terpenes in the milk of cows subjected to a similar supplementation of hemp and savory in their diet yielded distinct results. While both analyses involved the same supplementation, the differences in the chemical nature and metabolic pathways of terpenes and fatty acids may explain the disparate outcomes. Terpenes follow a distinct metabolic pathway. Terpenes are known for their rapid metabolism and can be volatile, leading to relatively quicker changes in their concentrations compared to fatty acids. Terpenes are volatile organic compounds synthesized through the mevalonic acid pathway in plants, and their presence in the milk may be more directly influenced by the chemical composition of the botanicals in the diet. The intricate terpene profiles observed in the milk could reflect a more immediate response to the supplemented hemp and savory, potentially due to the volatile nature of terpenes and their rapid absorption and distribution in the body (Arruda et al., 2013). This contrast underscores the divergent metabolic fates and responsiveness of terpenes and fatty acids to dietary interventions, elucidating the need for tailored approaches in studying different compound classes within the realm of nutritional sciences. In summary, the observed differences in the two studies can be correlated to the distinct characteristics of fatty acid and terpene metabolic pathways. The fatty acid metabolism may exhibit greater stability and resilience to modest dietary changes, while terpenes, being more volatile, could showcase noticeable variations even at lower concentrations in response to dietary interventions. Understanding these pathways provides valuable insights into the intricate mechanisms governing the composition of milk constituents in the context of dietary modifications.

### 4.5.3 Pasture altitude and dairy product quality

According to Coppa et al. (2019), semi-natural pasture has also recently emerged as a critical component of dairy product quality and traceability. It is well established that pastures with a variety of botanical species impart unique nutritional and organoleptic properties to milk, as opposed to concentrate feed made primarily of grains or preserved forages. The major vegetation type consumed by dairy animals has an impact on the chemical composition of milk and dairy products, especially in a pasture-fed dairy production system, which influences the milk's sensory qualities and nutraceutical qualities (Coppa et al., 2019). Summer mountain grazing produces alpine products with distinct qualities, particularly in terms of higher concentrations of certain fatty acids (FAs) thought to be good for human health. It has been consistently demonstrated that grazing, especially at higher altitudes, is a significant contributor to dairy products that have higher concentrations of branched-chain FAs, rumenic acid (*c*9, *t*11 C18:2), and a-linolenic acid (C18:3 n-3) (Cifuni et al., 2022). In the study conducted by Cifuni et al. pasture altitude significantly impacted the percentage

of nearly all FAs. The predominant fatty acids (FAs) in all experimental groups were palmitic acid (C16:0), oleic acid (C18:1 *c9*), stearic acid (C18:0), and myristic acid (C14:0), together comprising almost 75% of the total milk FA. The notably high content of milk PUFA from cows grazing in the highland pastures may be explained by differences in the botanical composition of the grass in the mountains compared to the lowlands, which alter the bacterial population in the rumen and lipid mobilisation of unsaturated FA due to energy shortage in the cows. Whereas terpenes are one of the more well researched volatile chemicals found in milk. They belong to a broad family of chemicals that are virtually entirely produced by plants and are found in animal tissues and products after being moved from pasture. (Cifuni et al., 2022) . Terpenes found in dairy products have drawn attention due to their potential effects on milk and, in turn, cheese qualities, as well as their ability to serve as indicators of the presence of a variety of forages in dairy cows' diets in both milk and cheese. Terpenes have been utilized for a while now as terroir markers to determine the provenance of dairy products like milk and cheese. Specifically, the composition of terpenes can offer valuable indicators for characterizing dairy products based on their place of origin and production circumstances (Engel et al., 2007).

### 4.5.4 Potential effects of terpene on rumen fermentation and milk quality

Ruminal biohydrogenation (BH) is a natural process in the rumen where UFA are converted into saturated fatty acids SFA. This is concerning because SFA can be harmful to human health. BH occurs due to microbial activity in the rumen (Thanh & Suksombat, 2015). BH of FA is a process that gradually alters linoleic acid (LA) and linolenic acid (LNA), both containing 18 carbon atoms. As this process unfolds, these acids transform into various intermediates with differing numbers of double bonds, ultimately leading to the production of stearic acid (SA). If this process can be regulated to prevent complete conversion of UFA into stearic acid, it could potentially improve the healthiness of meats and milk from ruminant animals (Vasta & Luciano, 2011). In this context, researchers have focused on the amount of PUFA, particularly conjugated linoleic acid (CLA), found in milk fat. CLA is naturally present in milk and is thought to positively influence various important bodily functions (Noni & Battelli, 2008). A recent study by Vasta and Luciano (Vasta & Luciano, 2011)demonstrated that plant secondary compounds (PSCs), particularly terpenes, can impact the concentration and production of fatty acids, in addition to their known antimicrobial and antibiotic properties. The inclusion of these compounds in an animal's diet has been linked to higher levels of polyunsaturated fatty acids (PUFA) in dairy items. This increase could be due to the influence of terpenes on biohydrogenation processes (Vasta & Bessa, 2012).

# Chapter 5

# Conclusion

In summary, our investigation aimed to explore the impact of hemp and savory supplementation on the aromatic profile of milk, with a specific focus on terpenes, as well as the dynamics of fatty acids. The study was guided by the recognition of the potential influence of dietary components on milk composition, which has implications for both animal and human nutrition. The findings of our study contribute to the understanding of how aromatic compounds, particularly terpenes, vary in response to dietary interventions. While the fatty acid composition of milk showed a notable stability across the experimental period, terpenes exhibited discernible variations. The objectives of the study were addressed through a robust experimental protocol, involving a Latin Square Experimental Design and comprehensive chemical analyses using advanced techniques such as Two-Dimensional Gas-Chromatography (GC × GC) and Headspace Solid-Phase Microextraction – Gas Chromatography-Mass Spectrometry (HS-SPME – GC-MS). The thorough examination of the chemical characteristics of hemp and savory leaves, coupled with the quantitative evaluation of aromatic compounds, provided a comprehensive foundation for the interpretation of our results. Furthermore, studies examining the impact of animal diet on the quality of dairy products have primarily focused on how nutrient intake and different feeding regimens affect the concentrations of key milk components, such as proteins and fat. These fluctuations can significantly influence cheese-making outcomes and contribute to specific sensory attributes, particularly texture (Coulon et al., 2004). Terpenes and fatty acids found in milk contribute to its sensory qualities and affect several aspects of processed cheese quality (Koczura et al., 2021). Recent indications propose that terpenes, originating from specific plants, might indirectly impact the sensory attributes of cheese by altering the dynamics of microbial ecosystems during cheese-making and aging. This hypothesis arises from observations suggesting a potential inverse relationship between terpenes and volatile compounds found in cheese, such as sulfur compounds resulting from microbial enzyme-driven protein breakdown. Alternatively, this correlation could stem directly from the incorporation of terpenes into cheese milk (Coulon et al., 2004). By analyzing the terpene profiles present in the milk and cheese, it becomes possible to trace the origin of the cheese back to the specific diet of the cows. Different types of herbs and grasses contain unique combinations and concentrations of terpenes, creating distinct terpene profiles in the resulting dairy products. Therefore, the presence of specific terpenes in the cheese can serve as a marker or fingerprint of the cow's diet, indicating whether the cow was fed fresh herbs or grazed on pasture (Fernandez et al., 2003).

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