EFFECT OF INCORPORATING ROSEMARY AND CLOVE EXTRACTS ON THE PHYSICOCHEMICAL, MICROBIOLOGICAL AND SENSORY CHARACTERISTICS OF SOFT CHEESE DURING STORAGE

by

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Effect of Incorporating Rosemary and Clove Extracts on the Physicochemical, Microbiological and Sensory Characteristics of Soft Cheese During Storage

A dissertation submitted to the Department of Food Technology, Central Campus of Technology, Tribhuvan University, in partial fulfilment of the requirements for the degree of B. Tech. in Food Technology

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Approval Letter

This ***dissertation*** entitles ***Effect of Incorporating of Rosemary and Clove extract on the Physicochemical, Microbiological, and Sensory Characteristics of Soft Cheese During Storage*** presented by **Sabina Acharya** has been accepted as the partial fulfillment of the requirement for **B. Tech. degree in Food Technology**.

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Abstract

The objective of the work was to study the effect of rosemary and clove extracts on physicochemical, microbiological and sensorial characteristics of soft cheese during storage for 28 days. Rosemary and clove extracts was prepared by ethanolic extraction methods. The antioxidant potential, total phenolic and flavonoid content of both extracts were determined. Soft cheese was prepared by incorporating varying concentrations (0%, 0.1%, 0.2%, 0.3%, 0.4% and 0.5%) of rosemary and clove extracts individually into the cheese curd. The best formulation was selected through sensory evaluation and analyzed for 28-day storage period with the interval of 7 days.

Clove extract exhibited higher total phenolic, flavonoid content and stronger antioxidant capacity compared to rosemary extract. Sensory analysis revealed that cheese with 0.2% rosemary extract and cheese with 0.1% clove extract were selected as the best cheese. During storage period, the moisture and pH levels of cheeses decreased while fat, protein, ash content and acidity increased. Free amino acids and free fatty acids increased at a slower rate in extract incorporated cheese samples compared to control cheese. Microbial analysis showed significantly lower total plate counts and yeasts and molds count in extract incorporated samples than control. A significant difference ($P<0.05$) was observed between control and extracts incorporated samples in terms of texture, taste and aftertaste. However, no significant differences ($P>0.05$) were found in other sensory attributes, including appearance, odor and overall acceptability during storage. Hence, incorporating rosemary and clove extracts effectively extended the shelf life and microbial safety of soft cheese, supporting their potential as natural, safe food preservatives.

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List of Abbreviations

Abbreviation	Full form
ADV	Acid degree value
ANOVA	Analysis of variance
AOAC	Association of official analytical chemist
BA	Biogenic amines
BHA	Butylated hydroxyanisole
BHT	Butylated hydroxytoluene
CFU	Colony forming unit
DDC	Dairy Development Corporation
DFTQC	Department of Food Technology and Quality Control
DPPH	2,2-diphenyl-1-picrylhydrazyl
FAA	Free amino acid
FAO	Food and Agriculture Organization
FDB	Fat on dry basis
FDM	Fat in dry matter
FFA	Free fatty acid
FRAP	Ferric reducing antioxidant power
GAE	Gallic acid equivalent
GDL	Glucono delta lactone
HSD	Honestly significant difference

Abbreviations	Full form
HTST	High temperature short time
IDF	International Dairy Federation
LAB	Lactic acid bacteria
LDPE	Low density polyethylene
LTLT	Low temperature long time
MCA	Milk clotting activity
MFFS	Moisture in fat free substances
PA	Protease activity
PCA	Plate count agar
PDA	Potato dextrose agar
TPC	Total phenolic content
UHT	Ultra-high temperature
UF	Ultrafiltration
VRBA	Violet red bile agar
WHO	World Health Organization

Part I

Introduction

1.1 General Introduction

Cheese is a fermented milk-based solid dairy product, which is produced by coagulating milk (whole, skimmed milk, or partially skimmed milk, butter milk or any combination of above materials) with the help of calf rennet or suitable coagulating agents (microbial or vegetable rennet) and partly draining the whey (Hickey, 2017). Milk used for the preparation of cheese can be obtained from cow, buffalo, camel, goat, yak, sheep, reindeer, horse, and donkey, as well as milk powder (Fox and McSweeney, 2017). Cheese is a highly nutritious fermented food. In comparison to whole milk, it contains 30-40% protein and fat, along with other minerals and fat-soluble vitamins (Khanal *et al.*, 2019).

Cheese is a nutrient-rich dairy product because they include nutrients that the body needs in high amounts, such as lactose, lipids, and milk casein, as well as rich source of protein, minerals like calcium and phosphorus, due to which it is very prone to microbial spoilage and subsequent biochemical deterioration (Al-Jubory and Habeb, 2019). Microbial growth and lipid oxidation is a major problem causing shelf life, quality deterioration in milk product like cheese especially during long term storage (Kristensen and Skibsted, 1999). Oxidation is responsible for off-flavors due to the formation of various volatile carbonyl compounds and may also degrade vitamins and β-carotene, which impair product quality and marketability (Day *et al.*, 1963).

Growing awareness and concern about the quality and safety of cheese have led to the development of new and improved methods for cheese preservation. Innovative preservation methods for ensuring safety and quality of cheeses include irradiation, low-temperature storage, vacuum packaging and addition of preservatives (Falih *et al.*, 2024). Synthetic preservatives like sorbic acid, sodium benzoate, nitrates etc. have long been used in food preservation, although some of them have suspected carcinogenic potential. Because of consumer concern over the safety of chemical and artificial preservatives, attention is shifting towards developing natural preservatives for the purpose of keeping food safe (Chauhan *et al.*, 2016). Currently, the addition of herbs or their extracts and essential oil is

widely accepted methods in food preservation and this trend of application can also be applied in cheese to extend its shelf life (Beuchat and Golden, 1989).

Extracts some herbs and plants were traditionally used for the preservation of wide variety of foods. Herbs and spices have been recognized to possess a broad spectrum of active constituents that exhibit antibacterial, antifungal, antiparasitic, and/or antiviral activities (Delsa, 2018). Similarly, spices and herbs are rich source of antioxidants. The antioxidant properties of herbs are due to presence of some vitamins, flavonoids, terpenoids, carotenoids and phytoestrogens (Shan *et al.*, 2011). Various herbs and spices, including basil, ginger, sage, moringa, pomegranate peel, rosemary, and cloves are known for their antioxidant and antimicrobial properties. Owing to these bioactive characteristics, they have been utilized as natural preservatives to enhance the shelf life of cheese (Sleem and Shaaban, 2023). The addition of spices and herbs to cheese can be carried out by incorporating them into milk (before cheese making), into cheese curd, or by rolling the cheese into crushed herbs (Dupas *et al.*, 2020).

Rosemary (*Rosmarinus officinalis* L.) known as an aromatic herb is being used as a food flavoring. Rosemary oil has traditionally and widely been used as a medicinal herb due to its anti-inflammatory, analgesic, astringent, antimicrobial, anti-rheumatic, carminative, antifungal, and antioxidant qualities (Oluwatuyi *et al.*, 2004). The extracts of rosemary leaves contain bioactive phytochemicals such as caffemic acid, carnosic acid, rosmarinic acid, and carnosol which are responsible for many of its well-known pharmacological effects. Among the phenolic components of rosemary, carnosol and carnosic acid account for over 90% of its antioxidant activity due to their inherent capacity to scavenge free radicals (Mohamed *et al.*, 2016).

Clove *(Syzygium aromaticum)* is a rich source of antioxidants and antimicrobial compounds, and can have an enormous potential for a food preservative. Clove buds is reported to have antibacterial, antifungal, antioxidant, antitumor, anti-inflammatory, insecticidal and flavor imparting characteristics (Ishaq *et al.*, 2019). Clove extract is rich in eugenol, a bioactive compound with known antimicrobial which significantly reduce the microbial counts (Armah *et al.*, 2025) and antioxidant properties that reduce lipid oxidation in cheese during storage, thereby enhancing shelf life (Pandey *et al.*, 2022). Thus, the present

study is focused on the utilization of natural herbs and spices i.e. rosemary and cloves as a preservative in cheese.

1.2 Statement of the problem

Cheese is a highly nutritious food that is susceptible to contamination by pathogenic and spoilage microorganisms, which can result in a reduced cheese shelf life, development of off flavors as well as in risks to the consumers health (Robinson, 2005). Although many cheeses undergo a heat treatment during the cheesemaking process, cheese is generally susceptible to contamination by microorganisms, which can result in cheese spoilage, health risks for consumers, and reduction in the cheese shelf life (Ritota and Manzi, 2020). Various methods for increasing shelf life of cheese have been made such as addition of preservatives, modified atmospheric packaging, active coating, edible coating and combination of them (Jalilzadeh *et al.*, 2015).

Food additives such as sorbic acids and sorbates, acetic acid, lactic acid, lysozyme, propionic acid and propionates, natamycin, hexamethylene tetramine and nitrates have been used as a preservative to extend the shelf life of the cheese. Although these preservatives are safe for human health when used in the recommended dosage, but they are extensively used in the food industry, the consumption of a large amount of these additives may result in certain health issues (Abdulmumeen *et al.*, 2012). As a result of increasing concerns about the safety of some chemical preservatives and consumers' negative perceptions of synthetic additives, there is a growing interest in more natural alternatives, including plant-based substances (Rao and Chauhan, 2024). Rosemary and clove extracts are abundant in bioactive compounds, especially polyphenols, which exhibit strong antioxidant and antimicrobial activities, positioning them as promising natural preservatives for use in dairy products (Nieto *et al.*, 2018; Rathod *et al.*, 2023).

In Nepal, people are not aware about the importance and benefits of the rosemary and those farmers with the knowledge of rosemary’s potentials, face challenges due to low limited market access, leading to its primary use as incense and underutilization in food. Despite the proven efficacy in international studies, the application of rosemary and clove extracts in dairy products remains largely unexplored in Nepal. Most local cheese producers continue to rely on conventional preservation methods, which may not adequately address concerns related to shelf life, microbial safety, and consumer preference for clean-label

products. Investigating the effects of these extracts on cheese can provide valuable insights into improving product quality, extending shelf life, and promoting the use of plant-based functional ingredients in dairy sector.

Hence, this study is carried out to develop the cheese incorporating rosemary and cloves extracts and to study their influence on physicochemical, microbiological and sensory properties of cheese during storage.

1.3 Objectives of the study

1.3.1 General objective

The general objective of this work is to study the effect

of incorporating the extracts of rosemary and clove on the quality and shelf life of soft cheese, focusing on its physicochemical, microbiological and sensory characteristics.

1.3.2 Specific objectives

The specific objectives of this work are as follows:

1. To prepare the ethanolic extract of rosemary and cloves and determine its extraction yield.
2. To determine the total phenols, flavonoids and antioxidant activity of extracts.
3. To prepare soft cheese with the incorporation of varying concentrations of rosemary and clove extracts separately.
4. To optimize the concentration of rosemary and clove extract in cheese based on the sensory evaluation.
5. To study the effect of rosemary and clove extract on physicochemical, free amino acid, free fatty acid, microbiological, and sensory quality of optimized cheeses during storage compared to control cheese.

1.4 Significance of the work

The present work might facilitate the promotion of use of natural ingredients in cheese to extend its shelf life. This might widen the possibilities for production and distribution of cheese as well as the subsequent utilization of natural herbs. The use of natural extracts as preservatives can increase the product's value, provide cost-effective benefits for manufacturers and may contribute to greater market availability (Maddaloni *et al.*, 2025). The use of natural extract would also offer the health benefits due to its antioxidant properties which can potentially enhance the nutritional profile of the cheese (Lee *et al.*, 2016). This would also attract health-conscious consumers who prefer food items made from natural ingredients instead of artificially produced compounds in their food. Rosemary and cloves are rich in phenols and thus acts as an antioxidant in body. Apart from this, the antimicrobial property of the herbs helps to replace or reduce the synthetic preservatives thus assuring food safety (Meng *et al.*, 2017). Since cloves and rosemary are available locally in Nepal, their use could be cheap alternatives for preservation of cheese while the use of such herbs would be promoted.

1.5 Limitations of the work

1. Proximate composition of rosemary and clove used was not determined
2. Antimicrobial potential of extracts was not studied.
3. Phytochemical analysis of cheese during storage was not conducted.

Part II

Literature review

2.1 Cheese

Cheese is a solid dairy product made from fermented milk that comes in a variety of flavors, textures and shapes and is manufactured all over the world (Singh *et al.*, 2003). It is a great food source of vitamins, minerals, and high-quality protein, including dietary calcium that is easily absorbed (Rana *et al.*, 2017). Cheese, in addition to being an effective way of preserving milk, has a long history. It is a food product that represents a community's cultural memory and history. Cheese types are influenced by the cultural structure of nations, climate conditions, animal variety, and production processes. Cheese is rich in milk fat and casein, which are retained in the curd during production. Water soluble components (whey protein, lactose, and water-soluble vitamins) are present in small amounts and largely partition into the whey. Manufacturing cheese has been passed down from generation to generation as an art form that has evolved over time into a gourmet food (Khetra *et al.*, 2016).

Cheese is a centuries-old cuisine with origins dating back before written history. There is no convincing evidence that cheesemaking began in Europe, Central Asia, or the Middle East, but the practice had spread throughout Europe before Roman times and had matured into a sophisticated process by the time the Roman Empire was created (Civitello, 2011). The earliest proposed dates for the origins of cheesemaking range from around 8000 BCE, when sheep were first domesticated. Since animal skins and inflated internal organs have been used as storage vessels for a variety of foodstuffs since ancient times, it is probable that the process of cheesemaking was discovered by accident by storing milk in a container made from an animal's stomach, resulting in the milk being turned to curd and whey by the rennet from the stomach (Silanikove *et al.*, 2015).

The primary reason for turning milk into cheese is to preserve a perishable food by converting it into a stable and storable product. Today, the major reason for cheese consumption is not hunger prevention, but the supply of essential nutrients, its various culinary applications, and its enjoyment (Molimard and Spinnler, 1996). Cheese is valued for its high fat, protein, calcium, and phosphorus content, as well as its portability and long shelf life. Cheese is more compact and has a longer shelf life than milk, yet how long a

cheese will last depend on the type of cheese. Hard cheese lasts longer than the soft cheese (Johnson, 2017).

2.2 Cheese production and consumption

In 2024, global cheese production amounted to about 22.52 million metric tons. The European Union was by far the top producer of cheese worldwide. In 2024, the 27 countries that makeup the European Union produced about 10.7 million metric tons of cheese. Europe has the highest level of per capita cheese consumption. In 2024, the European Union consumed about 9.5 million metric tons of cheese, an amount that far exceeded consumption number in other parts of the world. U.S. and Russia came in second and third in that year at about 6.1 and 1.4 million metric tons of cheese per capita (Shahbandeh, 2025).

Within the dairy industry, cheese is the most rapidly growing product. The rising intake of cheese can be attributed to a variety of factors, including a positive dietary image, ease and flexibility of use, and a wide range of flavors and textures (Cogan *et al.*, 2017).

2.2.1 Cheese production in Nepal

Dairy development activities in Nepal began after 1952. In 1953, the Food and Agriculture Organization (FAO) assisted in establishing a Yak cheese factory in Langtang, Rasuwa district, marking a milestone in Nepal's dairy development (Shingh *et al.*, 2020). A number of small scale cheese makers initiated production of Swiss Emmental and French Cantal type cheeses in Langtang and Ilam (Khanal *et al.*, 2019). Yak cheese is produced by the Dairy Development Corporation (DDC) and the private sector. Swiss technology is used in the production of seasonal cheese, which is produced for just seven months out of the year and is produced without interruption in factories for the five months between December and April. In four areas, there are approximately 21 private yak cheese manufacturers (Joshi and Bahadur, 2015).

The DDC produces four types of cheese in the public sector: Yak, Kanchan (cow), Mozzarella and Processed cheese. Sherpas make a very hard acidic cheese called "*Churpi*" in the private sector (Neupaney *et al.*, 1997). The Department of Food Technology and Quality Control (DFTQC) and other offices in Nepal have registered 53 small and medium-sized cheese industries (Khanal *et al.*, 2019). Six yak cheese factories are operating in four districts (Ramechhap, Dolakha, Solukhumbu, and Rasuwa), four kanchan cheese factories

are operating in two districts (Illam and Panchthar), and one buffalo-milk cheese factory is operating in Nagarkot (Kavrepalanchowk). DDC is responsible for all of these 8 factories (Thapa, 2006).

There are fewer varieties of cheese in Nepal than in Europe or America. The supply and consumption of cheese are rising in Nepal as a result of growing knowledge of the nutritional benefits of the dairy product as well as the development of cattle husbandry. Manufacturing cheese could be a tempting project to get past the milk holiday in the season of surplus. If cheese meets the quality standards established by regional, national, and international organizations, there can be a lot of opportunity for export (Khanal *et al.*, 2019).

2.3 Varieties and classification

Starting from a limited range of raw materials (bovine, ewe, goat, or water buffalo milk, starter cultures, coagulant i.e. rennet or acid and salt), there are large number (more than 1500) of cheese varieties (Cogan *et al.*, 2017). The majority of traditional classification schemes rely solely on one or more of the following factors: cheese composition, cooking temperature, coagulation method, milk type, textural properties (firmness), and characteristic ripening agent. Nonetheless, only a small number of classification models are based on integrative methods, which provide a more accurate picture of cheese's complexity and ability to distinguish between its numerous variations (Almena-Aliste and Mietton, 2014).

Khanal *et al.* (2019) stated that there are three different schemes of cheese classification and they are:

1. Based on texture,
2. Based on method of coagulation and
3. Based on ripening index.

However, International Dairy Federation (IDF) report lists the characteristics of cheese under the following headings (Upadhyay, 2003).

4. Country of origin.
5. Raw milk: cow, buffalo, sheep, goat.
6. Type of cheese: hard, semi-hard, soft, fresh, acid coagulated or whey cheese.

7. Internal characters: close or open texture, large medium or small eyes/holes, slit openings in curd blue or white mold ripened, color of curds.
8. External characters: rind hard, soft, smooth or rough, smear or mold ripened spices or herbal addition type of coating (plastic, ash).
9. Weight of cheese: shape and size.
10. Fat in dry matter (FDM)/Fat-on dry basis (FDB): Percentage minimum/maximum.
11. Water in fat free substances (WFFS)/ Moisture in fat free substance (MFFS).

Classification of cheese on the basis of texture as given by Codex Alimentarius, FAO/WHO, and Standard A6 has been shown in the Table 2.1

Table 2.1 Classification of cheese according to Codex Alimentarius

MFBB[1] (%)	Types	FDB[2] (%)	Types
<41	Extra hard	>60	High fat
49-56	Hard	45-60	Full fat
54-63	Semi-hard	25-45	Medium fat
61-69	Semi-soft	10-25	Low fat
>67	Soft	<10	Skim

Source: Scott *et al.* (1998)

MFFB[1] equals percentage moisture on fat free basis.

FDB[2] equals percentage of fat on dry basis.

Bamforth and Ward (2018) classified cheese on the basis of (i) composition, (ii) firmness, and (iii) maturation agents, which has been shown in the Table 2.2

Table 2.2 Classification of cheese on the basis of composition, firmness and maturation agent.

Types of cheese	Examples
1. Soft cheese (50-80% moisture)	
a. Unripened low fat	Cottage, Quark, Baker's
b. Unripened high fat	Cream, Neufchatels
c. Unripened stretched curd or pasta filata	Mozzarella, Scamorze
d. Ripened by external mold growth	Camembert, Brie
e. Ripened by bacterial fermentation	Kochkgse, Handkgse, Caciotta
f. Salt cultured or pickled	Feta- Greek, Domiati- Egyptian
2. Semi-soft cheese (39-50% moisture)	
a. Ripened by internal mold growth	Blue, Gorgonzola, Roquefort
b. Surface-ripened by bacteria and yeast (surface ripened)	Limburger, Brick, Trappist, Port, du salut, St paulin Oka
c. Ripened primarily by internal bacterial fermentation but may also have some surface growth	Miinster, Bel Paese, Tilsiter
d. Ripened internally by bacterial fermentation	Pasta Filata, Provolone, Low-moisture mozzarella
3. Hard cheese (39% moisture maximum)	
a. Internally ripened by bacterial fermentation	Cheddar, Colby, Caciocavallo
b. Internally ripened by bacterial fermentation (CO_2 production resulting in holes or eyes)	Swiss (Emmental), Gruyere, Gouda, Edam, Samsoe

c. Internally ripened by mold growth	Stilton
4. Very hard cheese (34% moisture maximum)	Asiago Old, Parmesan, Parmigiano, Grana, Romano, Sardo
5. Whey cheese	
a. Heat and acid denaturation of whey protein	Ricotta (60 % moisture)
b. Condensing of whey by heat and water evaporation	Gjetost (goat milk whey; 13% moisture), Myost, Primost (13-18 % moisture)
6. Spiced cheese	Noekkelost - cumin, cloves

Source: Bamforth and Ward (2018)

The classification of cheese on the basis of mode of coagulation has been shown in the Table 2.3.

Table 2.3 Classification of cheese groups on the basis of mode of coagulation

Group	Example
Rennet cheese	Most major international varieties
Acid cheese	Cottage, Quarg, Queso-Blanco
Heat/acid	Ricotta, Ziger
Concentration/crystallization	Mysost

Source: Fox *et al.* (2004)

2.4 Soft cheese

According to Robinson (2005), soft cheeses have at least 61% water in the fat-free cheese matter and 10-50% fat in the dry matter (FDM). The FAO/WHO defines soft cheese as having more than 67% water in the fat-free cheese matter. The literature's data on the composition of different soft cheeses show that their moisture contents range from 50 to

80%. The majority of soft cheeses are eaten fresh, thus it's critical that the milk or other dairy ingredients used in the production of soft cheeses are sufficiently pasteurized to reduce the possibility of food poisoning (Melo *et al.*, 2014).

The nutritional composition of soft cheeses varies depending on whether they are ripened or unripened varieties. Fresh, unripened cheeses, such as cottage cheese, are low in fat, calcium, moisture, and contain unfermented lactose (Buttriss, 2003). According to Bamforth and Ward (2018), different types of soft cheese has been shown in the Table 2.4

Table 2.4 Types of soft cheese

Soft cheese	Examples
Unripened low fat	Cottage, Quark, Baker's
Unripened high fat	Cream, Neufchatels
Unripened stretched curd or pasta filata	Mozzarella, Scamorze
Ripened by external mold growth	Camembert, Brie
Ripened by bacterial fermentation	Kochkgse, Handkgse, Caciotta
Salt cultured or pickled	Feta- Greek; Domiati- Egyptian

Source: Bamforth and Ward (2018)

2.4.1 Uses of soft cheese

Soft cheese has long been considered an ideal source of protein for those on weight loss diets because it can be made in a variety of low-fat forms, it is inexpensive, and it can be used in a multitude of recipes. In the United States, billions of pounds of cottage cheese are consumed annually, probably due to the public's perception of it as a healthy food (Kongo and Malcata, 2016).

2.5 Basic principle of cheesemaking

According to Nielsen and Ullum (1989), the basic cheesemaking principles are concentration, preservation and ripening.

1. Concentration: Coagulation, whey exudation (in cheese vat cutting, cooking, stirring, during pressing and during salting), evaporation during storage.

2. Preservation: pasteurization, concentration, acidification, salting, addition of saltpeter, surface treatment, cooling.

3. Ripening: changes in solids (protein, lactose, fat).

2.6 Retention figures

At the beginning, the three-dimensional casein network which is formed during coagulation encloses all the other milk constituents. When the coagulum contracts, water and the constituents dissolved in the water are squeezed out, whereas fat globules and bacteria retained in the fine-meshed casein network (Acharya, 2010). The retention figures of some milk constituents in cheese are shown in Table 2.5.

Table 2.5 Retention figures of some milk constituents in cheese

Milk constituents	Retention figures
Protein	~75% (not higher than 88%)
Fat	~88-95%
Lactose	~3-5%
Ash	~30-40%
Citric Acid	~10%
Bacteria	~90% concentrated in curd grains

Source: Acharya (2010)

2.7 Ingredients for cheese manufacturing

The essential ingredients for cheese-making are: milk, starter culture, additives, coagulating agents, color and salt.

2.7.1 Milk

Quality and type of milk directly affect the quality of cheese. The different animals contain different percentages of fat and protein. The number of caseins and fat in milk determines quality of cheese (Ong *et al.*, 2017). For instance, higher fat recovery takes place in cheese made from goat milk due to the presence of smaller fat globules (Lucey *et al.*, 1993). Goat milk has lower levels of α_{s1}-caseins than cow milk. Cow milk isn't suitable for some cheeses, such as Feta. However, it is good for making Cheddar cheese, a type of hard cheese that develops flavor through the breakdown of α_{s1}-caseins by the rennet enzyme (Hill and Kethireddipalli, 2013).

The ratio of fat to protein in milk, the milk's pH, and the amount of calcium are all important indicators of the quality of the cheese. These three variables can be altered based on the need and types of cheese (Cogan *et al.*, 2017).

2.7.2 Starter culture

Starter cultures are microorganisms (bacteria, yeasts, or molds) that are added to fermented milk products during their production. Lactic acid bacteria (LAB), such as *Lactobacillus* and *Streptococcus*, are the primary acid producers in cheese. In addition, other bacteria, yeast, and mold cultures are also added to cheese for specific purposes (Cogan *et al.*, 2007). Selection of the cultures depends upon the types of cheese. Cultures are added during cheese making for the following purposes:

- Development of acidity.
- Production of special flavors i.e. acetaldehyde or diacetyl flavor in the cheese.
- Diminishing the late gas blowing defect and food poisoning caused by growth of *Clostridium tyrobutyricum* in cheese.
- To achieve health benefit as probiotics (Hill and Kethireddipalli, 2013).

Other types of microorganisms used in specific kinds of cheese are referred to as secondary and adjunct cultures. The major species of starter cultures used to make cheese are *Leuconostoc sp*., *Streptococcous thermophilus, Lactobacillus delbrueckii* subsp*. lactis, Lactobacillus delbrueckii* subsp*. bulgaricus* and *Lactobacillus helveticus* (Fernandes, 2009).

2.7.3 Additives

Cheese production involves the use of chemical agents such as calcium chloride, phosphates, and sodium or potassium nitrate/nitrite (Gascoyne, 2016). Calcium chloride enhances the rate of clotting and decreases the quantity of rennet required. If vegetables or microbial coagulants are used in place of rennet, it also aids in maintaining the balance between soluble, colloidal, and complex calcium ions. For manufacturing some cheese varieties, milk is diluted with water to increase the disaggregation of casein micelles. Sometimes, casein becomes more soluble, resulting in more whey loss and lower cheese output. As a result, calcium salts are used to compensate for casein loss (Scott *et al.*, 1998). Potassium (or sodium) nitrate inhibits the growth of butyric (*Clostridium tyrobutyricum*) and spore-forming bacteria. It also helps suppress gas-forming butyric acid bacteria. However, possibility of formation of carcinogenic nitrosamines due to the reaction of nitrate and amino acids limits the use of nitrates in cheese (Cogan *et al.*, 2017). Phosphates have been employed as stabilizers in the form of sodium phosphates (Gascoyne, 2016). Nisin, an antimicrobial agent, is also used to control the gas forming bacteria in processed cheese Nisin, an antimicrobial agent, is also used to control the gas forming bacteria in processed cheese (Dusterhoft *et al.*, 2017). Acidulants (lactic or acetic acid) are used in some cheeses (e.g. Ricotta cheese) and very rarely, phosphoric acid is used in the case where pH of the final product should not exceed 5 (Gascoyne, 2016).

2.7.4 Coagulating agents

The addition of the rennet enzyme causes the milk to coagulate in cheese. There are three sources of this enzyme, which is used to coagulate cheese: animals, plants and microorganisms. Rennet is extracted from the stomach lining of the young calf (Cogan *et al.*, 2017). The aspartic proteinase enzyme chymosin is found in crude rennet extract (Ong *et al.*, 2017). Nowadays, rennet is produced using genetically modified microbes by fermentation (Hill and Kethireddipalli, 2013). Coagulating agents can be derived from many

vegetables, including *Cynara cardunculus, Ficus carica, Arctium minus*, and *Solanum dobium (*Alihanoglu *et al.,* 2018*).*

Rennet addition has two significant kinetic activities in milk coagulation. The first stage involves rennet's proteolysis of micellar casein, followed by the secondary stage when destabilized casein micelles combine to form the gel (Thomann *et al.*, 2008). Rennet plays a dual role in cheese production, coagulating milk and enhancing flavor and texture through proteolysis during ripening (Fox, 1998). Rennet coagulation is directly affected by the type of enzyme used, ratio and composition of the milk ingredients (fat, protein, whey and caseins), presence of calcium ion and pH (Cogan *et al.*, 2017). Rennet is most effective at 6.5 pH and 30^0C. Firm gel is only produced if there is free calcium (Ca^{2+}) ion available. Hence, ionic calcium (in the form of $CaCl_2$) up to 1mM is added to the cheese milk (Hill and Kethireddipalli, 2013).

2.7.4.1 Calf rennet

The most common milk clotting enzyme used in the cheese industry is calf rennet, which is predominantly extracted from the fourth stomach of nursing calves (Kethireddipalli and Hill, 2015; Ben Amira *et al.*, 2017; Mamo and Balasubramanian, 2018). Calf rennet has a high milk clotting activity/proteolytic activity (MCA/PA) ratio and is heat sensitive, resulting in high cheese yield (Moschopoulou, 2011). Calf rennet, the first milk clotting enzyme used in cheese production, has been recognized for its high specificity in releasing caseinomacropeptide from κ-casein (Liu *et al.*, 2021).

Calf rennet hydrolyzes the Phe_{105}-Met_{106} site of casein. The hydrophilic macropeptide and para-κ casein diffuse into the whey. More than half of κ-casein is hydrolyzed at pH 5.2-6.6 and 35^0C. Para-κ-casein forms a three-dimensional network micelle structure (Ghorbel *et al.*, 2003). Calcium ions act as a bridge between micelles, neutralizing the negative charge on their surfaces and causing them to aggregate and form curd (Liu *et al.*, 2021). Calf rennet is particularly expensive since it must be extracted from animal stomach tissues and then purified in a number of procedures (Adhikari *et al.*, 2021).

2.7.4.2 Microbial rennet

The manufacture of rennet from microbial and plant sources is receiving a lot of attention as the demand for cheese has increased globally over the past three decades and the supply of

calf rennet has reduced. Microbial sources of rennet production cost less due to their easy production through fermentation (Annapure and Gaur, 2022). Many microorganisms are known as producers of rennet such as proteinase, which can substitute the calf rennet. Microorganisms like *Rhizomucor pusillus, Endothia parasitica*, *Aspergillus oryzae*, and *Thermomucor indicae*- 18 *seudaticae* N31 are extensively used for rennet production in cheese manufacture (Silveira *et al.*, 2005). The aspartyl protease released by the fungus *Rhizomucor miehei* is the most effective type of microbial rennet that have been used as a substitute for almost 40 years (Jacob *et al.*, 2011). This enzyme has a high ratio of MCA/PA an important requirement to substitute calf rennet (Thakur *et al.*, 1990).

2.7.4.3 Plant rennet

Plant proteases hydrolyze protein molecules into peptides and amino acids. These constitute a diverse and complex group of enzymes, which differ in properties such as substrate specificity, active site and catalytic mechanism, pH and temperature, etc. The specificity of proteolytic enzymes is determined by the nature of the amino acid and other functional groups close to the bond being hydrolyzed (Sumantha *et al.*, 2006). Proteases are divided into groups on the basis of catalytic mechanism used during the hydrolytic process. The main catalytic types are aspartate, serine, cysteine, and metalloproteases (Shah *et al.*, 2014).

2.7.5 Color

Color is an important aspect in consumer preference for any food. There are two types of color which are very important in the milk (Wadhwani and McMahon, 2012). One is riboflavin, found in serum protein (whey) and another is carotene, found in milk fat of some animals. Most of the riboflavin is lost in whey during cheese manufacturing process and carotene (carotenoid) remains in cheese. The color of milk varies significantly during summer and winter. As a result, some cheese manufacturers add saffron or annatto to their products in an effort to preserve the color throughout the year (Scott *et al.*, 1998; Cogan *et al.*, 2017). Annatto and β-carotene are used in cheese colorings alone or in combination at 0.06% w/w. Annatto is used for the high fat cheese (Chapman *et al.*, 2007; Kang *et al.*, 2010). Carotene color is effective at a very low concentration only. Cheese made from carotene can become too yellow (Chapman *et al.*, 2007).

2.7.6 Salt

Role of salt in cheese manufacturing is very significant due to its preservative action. It also incorporates dietary sodium (to a lesser extent) and controls the growth of both starter and non-starter LAB, pathogenic and contaminant bacteria in cheese (Guinee and Fox, 2017). Salt imparts flavor to the cheese and checks the growth and metabolism of microorganisms. It also influences the texture of cheese (Kaya, 2002). Sodium chloride (NaCl) or salt is also associated with other functions, such as composition of cheese, solubility of protein, water activity, casein network hydration, calcium and para-caseinate complex interactions and curd syneresis (Johnson *et al.*, 2009; Guinee and Fox, 2017). The optimum level of NaCl in Cheddar cheese is from 1.8 to 2.1% (w/w) (Rulikowska *et al.*, 2013). Prasad and Alvarez (1999) studied the effect of salt on the physiochemical characteristics of Feta cheese throughout the ripening and illustrated that harder cheese was developed by a higher percentage of salt in brine. They also demonstrated changes in color of the cheese by varying salt concentration in the brine.

2.8 Manufacturing steps involved in cheesemaking

The production of all varieties of cheese involves a generally similar procedure, in which various steps are modified to give a product with the desired characteristics (Fox *et al.*, 2000) The general steps involved in cheesemaking are:

- Selection, standardization and, in most cases, pasteurization of the milk.
- Acidification, usually via the in-situ production of lactic acid by selected bacteria.
- Coagulation of the milk.
- Dehydration of the coagulum to yield cheese curd, by a range of techniques, some of which are variety specific.
- Forming the curds into characteristic shapes.
- For most varieties, ripening (maturation) of the curd during which the characteristic flavor and texture of the cheese develop.

2.8.1 Selection of milk

The cheese milk composition, including fat, protein, calcium, and pH, significantly influence cheese composition. Milk constituents are influenced by various factors such as animal

species, breed, individuality, nutritional state, health, and lactation stage. Milk from cows in early or late lactation, or those with mastitis, should be avoided due to compositional abnormalities. The number of somatic cells (leucocytes) is a good indicator of quality. Poor microbiological quality of milk can lead to contamination of cheese curds and public health issues (Cogan *et al.*, 2017).

2.8.2 Pretreatment of milk for cheesemaking

Various pre-treatments such as chilling, thermization, bactofugation standardization, pasteurization, filtration/centrifugation are performed to improve the characteristics of cheese.

2.8.2.1 Chilling

In a modern farm, milk is quickly chilled to below 8^0C and kept for one to three days before being collected. Furthermore, cold milk is transported across large distances and is often cold-stored at the cheese plant for 1-3 days, depending on the season and manufacturing schedule (McSweeney, 2007). Refrigerated storage of raw milk is widely used to minimize the growth of microorganisms in milk prior to processing. A number of biochemical changes can occur in chilled milk. Many organisms belonging to the genera Bacillus and Pseudomonas fluorescens are capable of producing lipases and proteases, which are able to hydrolyze milk proteins and fat. During raw milk storage at lower temperatures, the microflora of the milk changes when the population of psychrophilic bacteria exceeds 10^7 to 10^8/ml (Datta and Deeth, 2001).

2.8.2.2 Thermization

Thermization is a method of heating of milk at a temperature of around 57-65^{o}C for 15s that markedly reduce the number of spoilage bacteria in milk with minimal heat damage (Kilcawley *et al.*, 2007). Thermization renders psychotropic bacteria in milk inactive (Sun, 2005), enabling it to be stored for three days below 8°C or for seven days below 0–1°C (Early, 1998).

2.8.2.3 Bactofugation

Bactofugation is a centrifugal process for removing spores of microorganisms from milk that have low pasteurization. This involves removing the spores of *Bacillus cereus*, *Clostridium tyrobutyricum* and related types of species spore from the milk intended for cheesemaking (Jovanovska *et al.*, 2017). The majority of the spores' range in size from 1 to 1.5µm, but their density differs from that of bacteria. As a result, at temperatures for separation between 60 and 65^0C, a significant percentage typically 90-95% is eliminated (Walstra *et al.*, 2006). Spores are eliminated by centrifugation, yet around 3% of the components of milk are lost in the process. It is used to extend the shelf life of pasteurized milk, although it is a costly procedure that needs two separators in series to remove 99% of spores (Haenlein and Park, 2013).

2.8.2.4 Standardization

Standardization normally means adding skim milk or skim milk solids, or removing cream to increase the ratio of protein to fat (P/F) (Goff, 2003). Standardization can remove any major imbalance in milk composition and thereby assist in maintaining the uniform quality of a cheese (Phelan, 2007). Standardization of cheese milk controls the composition and particularly the fat in dry matter ratio of the cheese, maximizes cheese yield and helps to control cheese quality (Ottogalli *et al.*, 2017). Different cheese varieties have a characteristic fat in dry matter content (Fox *et al.*, 2000).

2.8.2.5 Pasteurization

Pasteurization of milk is performed to kill all pathogenic and harmful microorganisms and to inactivate phosphatase and xanthine oxidase enzymes present in the milk. Pasteurization also increases the yield of cheese by insolubilizing part of the serum protein (Banks, 2011). There are three systems of pasteurization practiced in most countries namely: flash heating (no holding) to temperature of 75-95℃, HTST: 71-75℃/14-40 s and LTLT: 61-65℃ / 20-40 min (Scott *et al.*, 1998).

2.8.2.6 Filtration/centrifugation

Removal of dirt particles is done by filtration or centrifugation. Bactofugation is sometimes applied to reduce the number of spores of *Clostridium tyrobutyricum* (to about 3%). The

removal of the sediment obtained, containing the spores, causes about 6% reduction in cheese yield. Therefore, the sediment is UHT heated and added again to the cheese milk (Narvhus *et al.*, 2007).

2.8.3 Conversion of milk to cheese curd

After standardization, pasteurization, or other treatment, the milk is adjusted to a temperature between 30 and 35°C, depending on the variety. It is then transferred to vats, also known as kettles, which can be open or closed, have a capacity ranging from a few hundred liters to 30,000 liters or more, and are shaped like hemispherical, rectangular, vertical, or horizontal cylinders (Legg *et al.*, 2017). Here, the milk is turned into cheese curd by a three-step process that involves acidification, coagulation, and dehydration (Law and Tamime, 2011).

2.8.3.1 Acidification

The acidification of milk is the crucial step in cheesemaking. Acidification enhances flavor and texture, facilitates coagulation, and reduces the growth of pathogens and spoiling organisms by reducing pH. It is normally obtained from the fermentation of lactose by bacterial starter cultures to produce lactic acid, although some fresh cheeses, such as cottage cheese, can be acidified by the direct addition of acid and do not require starter (Coelho *et al.*, 2022). In the past, the microflora in milk was responsible for acidification. However, this process is difficult to control and tends to give a variable product that may suffer from taints and inconsistent flavors. As a result, most cheeses are now made using selected starter, that gives predictable and desirable results. *Lactococcus lactis, Streptococcus thermophilus, Lactobacillus helveticus* and *Lactobacillus bulgaricus* are the primary species of starter bacteria used in cheese manufacture (Fernandes, 2009).

Direct acidification method involves addition of lactic acid/phosphoric acid to cold milk (2°C–12°C) to achieve a pH of 5.2 followed by addition of glucono-δ-lactone (GDL), which is slowly hydrolyzed to gluconic acid, resulting in a gradual reduction in pH to 4.6-4.8 (Makhal *et al.*, 2014). Direct acidification is more controllable than biological acidification, and, unlike starters, it is not susceptible to phage infection. In addition to acidification, the starter bacteria help in cheese ripening, and hence chemical acidification is used mainly for cheese varieties for which texture is more important than flavor (Fox *et al.*, 2000).

2.8.3.2 Coagulation

Most cheese makers choose optimum pH for the addition of rennet to coagulate the milk as pH governs the nature and speed at which the coagulum is formed (Ong *et al.*, 2017). Rennet is added to the acidified milk when it reaches pH 5.4-5.6 at 31- 35ºC. After the coagulum is completely formed, the curds are stirred and cooked with whey to a temperature of about 60-70ºC (Phadungath, 2005). The coagulation process must be brief, cost-effective, and well-regulated and should not cause excessive loss of curd and fat in whey. Generally, one part of rennet can clot 10,000 parts (0.01%v/v) of milk but it depends on concentration and manufacturer instructions (Legg *et al.*, 2017).

2.8.4 Curd treatment

The purpose of curd treatment in the cheese vat after coagulation is to promote the contraction of the casein network and resulting whey exudation (syneresis) without losing too much fat and curd in the whey (Harper and Hall, 1976).

2.8.4.1 Cutting the coagulum

Cutting time is very important during cheesemaking as it directly influences the yield and composition of cheese (Ong *et al.*, 2017). After a few minutes of rennet addition, the precipitation of para-κ-casein takes place. At the beginning, the coagulum is very soft but it gradually becomes firmer. The coagulum hardens faster at higher temperature and lower pH values. The rennet coagulum is cut (horizontal and vertical) with cheese knives into pieces, thereby, increasing the surface area of the curd for easy expulsion of whey. The mode of cutting the coagulum varies with the variety of cheeses, as it directly determines the rate and extent of moisture removal and the final cheese texture. The smaller the size of the curd piece, the greater is the moisture expulsion and harder is the texture (lower moisture) of cheese obtained (Acharya, 2010).

2.8.4.2 Cooking the curd

The combination of heat and the developed acidity (decreasing pH) causes syneresis with the consequent expulsion of moisture, lactose, acids, soluble minerals and salts, and whey proteins (Goff, 2003). Cooking temperature in cheese vat influences moisture removal from curd during cheesemaking and differences in moisture content could be a significant impact

on cheese. Thus, differences in cooking temperature may affect chemical composition and sensory characteristics of cheese (Abdalla and Mohamed, 2009).

2.8.4.3 Whey drainage

The process is performed to separate whey from curd and to aggregate or coalesce the curd particles. It takes about 15 min. The pressing is a traditional approach in which all the curd mass was collected in a cheese cloth or scooped into perforated molds to separate it from whey (Acharya, 2010). The whey is drained when the pH reaches 6.1-6.5 depending upon cheese variety (Tunick, 2014).

2.8.5 Salting

Variation of salt concentration in cheese is due to the method of application and the types of cheese. Immersion in brine solution and dry salting are the common practice for untextured cheese. Further ripening or ageing and proteolysis of the cheese are governed by salting (Ong *et al.*, 2017). Salting method varies according to the type of cheese. Salting on the surface of molded curd is done for blue veined cheese, brine salting is done for Edam, cheese is directly immersed in brine for Gouda cheese, and dry salting is done for cheddar and cottage cheeses. Cheese salted by brine are held to make the curd into a compact mass of appropriate size so as to handle it easily (Guinee and Fox, 2017).

The salt content directly affects the taste of cheese, provides sodium, which is important for regulating blood pressure and safe body cell function, and serves as a preservative. Salt decreases the activity of water inside the cheese matrix and consequently regulates the growth of bacteria, the activity of enzymes, the level of protein hydration and aggregation, and the rheological and cooking properties of cheese (Mijan *et al.*, 2010).

2.8.6 Ripening

Ripening is concerned with the production of desirable texture and aroma in cheese and is governed by the proteolysis and other different biochemical reactions in cheese (Cogan *et al.*, 2017). Ripening can be separated into two stages. The first stage is from 7 to 14 days, in which reduction in the rubbery texture of cheese is brought by the residual coagulant enzymes by rapidly hydrolyzing α_{s1}-caseins to α_{s2}-caseins degradation products (Lawrence *et al.*, 1987). During the residual ripening period, proteolysis is continued by coagulant

enzymes, native milk protease and enzymes produced by starter bacteria and secondary micro-flora (Cogan *et al.*, 2017). For cheese which is not vacuum packed, the temperature and relative humidity ranges from 5 to 12ºC and 87 to 95% (Hort and Le Grys, 2001).

2.8.6.1 Lipolysis of fatty acids

Lipolysis has a significant impact on cheese texture and flavor (Voigt *et al.*, 2012). Cheese lipases originate from milk, rennet, starter, auxiliary starter, nonstarter bacteria, and exogenous enzymes. Lipoprotein lipase plays a crucial role in developing flavor in raw milk cheeses, but has little impact on pasteurized cheese (Sert *et al.*, 2014). The lipolytic enzymes present in LAB can hydrolyze a substrate to generate free fatty acid esters, triacylglycerides, diacylglycerides, and monoacylglycerides (Holland *et al.*, 2005).

Fatty acids greatly influence the flavor of cheese. During cheese fermentation and ripening, fatty acids with medium and short carbon chains ($C < 4$) are produced through milk fat breakdown. This leads to the formation of characteristic flavor substances in cheese, that determine the maturity of the cheese. Oxidation of fatty acids, particularly polyunsaturated ones, can result in the production of flavorful unsaturated aldehydes. This can result in a rancidity-related unpleasant odor that is noticed when Gouda, Cheddar, and Swiss cheese deteriorate (Forde and Fitzgerald, 2000). However, lipolysis is beneficial to the majority of cheeses, including blue cheeses, Parmesan, Emmental, and Italian (such Romano) cheeses (Zheng *et al.*, 2021).

Fatty acids produced via lipolysis, especially free fatty acids such as acetic, octanoic, and decanoic acids, are cheese flavor substances. The flavors produced by fatty acids vary according to the differences of fatty acid types and contents in the many cheese types (Mallatou *et al.*, 2003). Butyric acid is an important flavor compound in cheese such as Romano and Cottage cheese, whereas the main characteristic flavor of Swiss cheese is propionic acid produced by propionic acid bacteria (Singh *et al.*, 2003). Moreover, hexanoic acid is responsible for a sweaty, pungent, and rancid flavor (Zheng *et al.*, 2021); octanoic acid imparts a goaty, waxy flavor; and decanoic acid affords a fatty, citrus odor (Gan *et al.*, 2016).

2.8.6.2 Proteolysis

Protein hydrolysis is a key biochemical reaction that influences the release and taste of cheese flavor during the ripening process (Gan *et al.*, 2016). Protease in cheese decomposes protein to produce peptides and free amino acids, which are precursors of many flavor compounds. Casein hydrolysis is the primary metabolic mechanism for flavor development in hard and semi-hard cheeses. Proteinases and peptidases cleave polypeptide chains, releasing free amino acids which act as precursors for flavor components during cheese manufacture and ripening (McSweeney, 2011).

Free amino acid content and metabolism in mature cheese play essential roles in cheese flavor development. Free amino acids in cheese undergo deamination, amination, and decarboxylase reactions resulting in a variety of volatile and nonvolatile flavor compounds (such as ketones, aldehydes, acids, and alcohols) under the influence of transaminase, deaminase, and other enzymes (Fox and McSweeney, 1998). The concentrations of methionine, leucine, and glutamic acid indicate cheese's level of protein hydrolysis. Enzymatic removal of amino acids produces flavors and aromas, including 3-methyl-butanol, methionyl-propyl aldehyde, sulfides, and aromatic esters. These compounds have malty, baked potato-like, pungent, and flowery aromas, respectively (Suzuki-Iwashima *et al.*, 2020).

Methionine in cheese is converted to volatile sulfur compounds such methanethiol, which has a rancid odor, and dimethyl sulfide and dimethyl trisulfide, which have a garlicky flavor; they represent the basic flavor substances in many cheese varieties (Smit *et al.*, 2005). S-Compounds are the primary source of the characteristic aroma of cheddar cheese and are also responsible for the garlicky smell of well ripened Camembert (Sousa-Gallagher and McSweeney, 2000). Apart from flavoring ingredient, low-molecular-weight biogenic amines can be produced by proteolysis and oxidative decarboxylation of amino acids; an excess of them can result in unfavorable physiological effects. Therefore, biogenic amines detection is a necessary part of cheese safety analysis (Spano *et al.*, 2010).

2.9 Herbs and spices

Botanically speaking, herbs are soft-stemmed plants whose main stems die to the ground and either never grow again (annuals), only grow again the next year (biennials), or regrow every

year (perennials). Herbs can be used either fresh or dehydrated. Despite having comparatively little amounts of essential oil, herbs generally have a mild and extremely distinct aromatic character (Reineccius, 2005). Spices are dried fruits, seeds, roots, bark, or other vegetative materials that are added to meals in small amounts for flavor, color or as a preservative, usually at nutritionally insignificant amounts (Rathor and Shekhawat, 2008).

Herbs and spices have been a vital part of human life for thousands of years and are utilized as flavoring, preservation, and coloring agents in nutraceutical, pharmaceutical, and cosmetics goods (Dini, 2018). Herbs and spices have shown numerous kinds of health benefits in the prevention and treatment of a wide variety of diseases, including metabolic, neurological, cardiovascular, cancer, and inflammatory diseases (Gottardi *et al.*, 2016). Also, herbs and spices have been utilized as food additives, not only to enhance the organoleptic properties of food, but also to increase the shelf life by decreasing or eliminating the foodborne pathogens (Lai and Roy, 2004).

Herbs and spices contain high amounts of bioactive compounds which possess natural antioxidants and potential antimicrobial activity. Plant-derived essential oils can inhibit the growth of microorganisms such as bacteria, yeasts, and molds that cause food spoilage and delay the development of rancidity. From chemistry point of view, they consist of aromatic and volatile compounds which play an important role in plant defense and possess antimicrobial properties (Hyldgaard *et al.*, 2012; Barak and Mudgil, 2023). Antimicrobial properties of herbs and spices can be successfully used to control the growth of spoilage and pathogenic bacteria in dairy products (Tajkarimi *et al.*, 2010).

2.10 Functional properties of herbs and spices

Spices and herbs have been used to enhance flavor, color, aroma, and food preservation. The consumption of herbs and spices has been shown to have health benefits. Herbal foods are recognized for their antimicrobial, antioxidant, nutritional, and therapeutic benefits. Herbs and spices contain bioactive elements such as carotenoids, coumarins, curcumins, flavonoids, lignans, phenolic acid phthalides, polyphenols, saponins, sulfides, terpenoids, and plant sterols. These bioactive components have been shown to have several biological effects such as antimicrobial, anti-inflammatory, antioxidant, antiallergic and antihypertensive effects (Paswan *et al.*, 2021). The main functional properties of the herbs and spices are discussed below:

2.10.1 Antioxidant properties

Antioxidants are substances that prevent or slow down the oxidation process. The food industry frequently uses synthetic antioxidants, such as butylhydroxyanisole or butylhydroxytoluene, to stop food quality deterioration (including the breakdown of lipids, carbohydrates, and proteins). However, these antioxidants are volatile and quickly break down at high temperatures, so consuming them could be extremely dangerous to health (Barak and Mudgil, 2023).

In contrast, herbs and spices are rich in bioactive substances that work as natural antioxidants. These natural antioxidants prevent food from spoiling by delaying the onset of rancidity when they are added to food products. By giving free radicals a hydrogen atom, bioactive compounds found in spices and herbs primarily function as scavengers of free radicals. Due to safety concerns with synthetic antioxidants, customers who are health-conscious are increasingly in demand for these naturally occurring antioxidants derived from herbs (Barak and Mudgil, 2023).

They act as antioxidants by scavenging free radicals and protecting the body from a variety of illnesses, including infections, diabetes, cancer, asthma, and cardiovascular disease. Numerous researchers have studied the various anticancer effects of herbs through mechanisms like increasing endogenous enzymes that prevent cancer, inhibiting the synthesis of nucleic acids, protecting DNA from structural damage caused by free radicals, inducing apoptosis, and preventing tumor growth. Meanwhile, the anti-diabetes and anti-thrombotic properties of herbs protect the cardiovascular system (Jain *et al.*, 2018).

2.10.2 Antimicrobial properties

The antimicrobial properties of the herbs have been demonstrated to be useful in extending the shelf life and stability of dairy products by inhibiting the growth of microorganisms that cause spoiling**.** There are several herbs and spices that have antimicrobial properties that work against various yeasts, bacteria, and molds (Benkerroum, 2013; Pisoschi *et al.*, 2018). In the 1880s, the first scientific investigation on the preservative effects of spices discovered that cinnamon oil had antibacterial properties against *Bacillus anthracis* spores (Campelo *et al.*, 2019). Different types of spices have been categorized based on their antimicrobial activities into strong (cinnamon, clove, mustard), medium (bay leaf, caraway,

coriander, cumin, oregano, rosemary, sage, thyme), and weak (black pepper, red pepper, ginger) (Mahmud and Khan, 2018).

The active components present in the herbs and spices include phenols, alcohols, aldehydes, ethers, ketones, and hydrocarbons. Oleuropein, tea catechins, ellagic acid, ferulic acid, and coumaric acid are only a few of the phenolic compounds that make up the majority of the antibacterial substances found in herbs. These can successfully take the place of synthetic antimicrobials in food products. *Salmonella enteritidis*, *Staphylococcus aureus*, and *Listeria monocytogenes* are just a few of the prevalent harmful bacteria that these phenolic compounds have been discovered to effectively suppress (Youssef and El-Sayed, 2019). Further, the essential oils present in herbs also contain certain bioactive compounds that show antimicrobial properties. Further, the essential oils present in herbs also contain certain bioactive compounds that show antimicrobial properties (Guine and Goncalves, 2016).

2.11 Application of herbs as preservative in functional dairy products

2.11.1 Ghee (clarified butter)

Deshmukh *et al.* (2018) studied the herbal ghee made from ethanolic extracts of *Asparagus racemosus (Shatavari)* and *Withania somnifera (Ashwagandha)*. Adding these herbs to ghee at varying concentrations improved its phytosterol content. Ethanolic extracts of *Asparagus racemosus* and *Withania somnifera* can improve the phytosterol content of ghee and act as antioxidants. The addition of coriander extract to ghee improves the antioxidant activity of ghee and offers better oxidative stability to ghee during storage compared to control ghee (Patel *et al.*, 2013).

Merai *et al.* (2003) observed that ghee prepared with 0.6% *Tulsi (Ocimum sanctum)* leaves extract was as stable as ghee with 0.02% butylated hydroxyanisole (BHA) after 8 days of high temperature storage (80 ± 2°C). Sage and rosemary extracts are commonly used to increase the shelf life of ghee and butter oil (Ozcan, 2003). These extracts have significantly higher antioxidant activity than synthetic antioxidants such as Butylated hydroxytoluene (BHT) and BHA (Estévez *et al.*, 2007).

2.11.2 Ice cream

Gremski *et al.* (2019) formulated antioxidant-rich ice cream using herbal extracts and fructo-oligosaccharides from *Ilex paraguariensis, Melissa officinalis, and Cymbopogon citratus*. In vitro studies revealed that the extract has antidiabetic, antihypertensive, and antioxidant properties. It also increased the phenolic content and antioxidant activity of the herbal ice cream. Ali *et al.* (2014) prepared herbal ice cream by incorporating powdered herbs (pomegranate, salep orchid, and asparagus) into the ice cream. The ice cream's antioxidant qualities were considerably enhanced by the addition of herbs (4%), as demonstrated by the 2,2-Diphenyl-1-picrylhydrazyl (DPPH) and Ferric reducing antioxidant power (FRAP) activities. Trivedi *et al.* (2014) recommended incorporating basil juice at the rate of 6% and freeze-dried basil powder at the rate of 1% by weight of ice cream mix in the preparation of 'basil flavored herbal ice cream'. Basil variety (*Ocimum sanctum)* was preferred over *O. americanum*, *O. basilicum* and *O. gratissimum*. Incorporation of basil juice led to decrease in fat, protein, carbohydrates, ash and acidity and an increase in pH; melting resistance of ice cream was reduced.

2.11.3 Yogurt

Ghalem and Zouaoui (2013) incorporated *Rosmarinus officinalis* oil to yoghurt at concentrations of 0.14, 0.21, 0.29, and 0.36 g/l and stored it for up to 21 days. The herbal yoghurt enhanced with 0.14 g/l of the oil received the highest score for taste, flavor, and texture from the panel. Yogurt containing beetroot extract and tulsi added at a rate of 5% had increased levels of nutrients. The addition of tulsi and beetroot extract increased the yogurt's riboflavin and folic acid levels. Furthermore, yoghurt infused with tulsi exhibited enhanced antiradical activity (Ghosh, 2019).

Yangilar and Yildiz (2017) found that adding ginger and chamomile essential oils (0.2% and 0.4%, respectively) to yogurt increased its preservation activity due to their antibacterial and antioxidant properties. Maji *et al.* (2018) found that adding turmeric to lassi (Indian yoghurt) at 1% (v/v) resulted in a higher phenolic content and sensory-acceptable lassi for up to 9 days when stored at 7°C in a glass bottle.

2.11.4 Butter

The impact of incorporating cinnamon extract on the functional properties of butter was studied by Vidanagamage *et al.* (2016). According to their findings, 3% cinnamon extract reduced the microbial count, peroxide, and free fatty acids in butter when compared to the control. Ozkan *et al.* (2007) studied the antioxidant activity of essential oil of *Satureja cilicica* in butter. The essential oil of *S. cilicica* showed high antioxidant activity when added to butter at 0.5, 1.0%, and 2.0% concentrations. Study found that *S. cilicica* essential oil can act as a natural antioxidant and aroma enhancer in butter. Sour cream butter containing 2% sage or rosemary exhibited improved oxidative stability and lesser secondary oxidative products, such as malonaldehyde and ketones, compared to the control butter during storage. However, rosemary herb was found to be more effective than sage in reducing lipolysis in butter (Najgebauer-Lejko *et al.*, 2009).

2.11.5 Paneer

Buch *et al.* (2014) incorporated turmeric into paneer coagulum at 0.4% and 0.6% of the expected yield. Paneer samples with 0.6% turmeric by weight lasted 12 days compared to 7 days for paneer without turmeric when stored at 7±1°C. When added to milk (0.6% by weight of expected yield) before heat treatment in the paneer-making process, turmeric powder helped to reduce the raw turmeric's taste in the finished product.

Similarly, Rajarshibhai (2012) studied the effect of selected spices (oleoresins and essential oils) of cardamom, clove and black pepper to extend the shelf life of paneer and has shown an extended shelf life of paneer prepared with 0.01% essential oil of cinnamon, 1:1 combination of essential oil of cinnamon plus cardamom and essential oil of cardamom alone to 10, 15 and 15 days respectively as compared to 5 days in control paneer at storage temperature of 7°C.

2.12 Rosemary (*Rosmarinus officinalis* L.*)*

Rosemary (*Rosmarinus officinalis* L.*)* is an aromatic evergreen shrub with the needle-like leaves, native to the Mediterranean region. It belongs to the Lamiaceae family. The plant blooms in summer and spring in temperate climates, but the plant can be in constant bloom in warm climates; flowers are white, pink, purple or deep blue (Khairnar *et al.*, 2023). It

usually grows to about 1 m tall, although some plants can up to 2 m tall. They are dark green and shiny above, with white undersides and curly leaf edges (al-Sereiti *et al.*, 1999).

Rosemary leaves are usually used as a seasoning to enhance foods, but this plant has also been used for medicinal purposes. In traditional medicine, rosemary has been used as a stimulant and mild analgesic, and it is regarded as one of the most efficient herbs for treating headaches, poor circulation, inflammatory disorders, and physical and mental exhaustion (Yu *et al.*, 2013).

Rosemary extracts have been utilized to treat diseases due to their hepatoprotective properties (Raskovic *et al.*, 2014), therapeutic potential for Alzheimer's disease (Habtemariam, 2016), and antiangiogenic effects (Kayashima and Matsubara, 2012). However, they have been used in food preservation to prevent oxidation and contamination from microbes (Djenane *et al.*, 2002; Nieto *et al.*, 2011). As a result, rosemary extract could be effective for substituting or even reducing synthetic antioxidants in diets. As preservatives, rosemary extracts provide consumers with a variety of technological advantages and benefits (Nieto *et al.*, 2018).

The EFSA (European Food Safety Authority) has investigated the safety of rosemary extracts. The study found that carnosol and carnosic acid intake is high, with estimations ranging from 0.09 (the elderly) to 0.81 (children) mg/kg/day. In the European Union, rosemary extracts can be added to food and drinks at up to 400 mg/kg (Aguilar *et al.*, 2008).

2.12.1 Chemical composition of rosemary extract

The primary constituents of rosemary's polyphenolic profile include hesperidin, carnosic acid, carnosol, and rosmarinic acid (Tai *et al.*, 2012). The cyclic diterpene diphenols carnosolic acid and carnosol have been found to be among the most potent antioxidant components of rosemary. Its extract also includes isorosmanol, methylcarnosate, carnosic acid, and epirosmanol (Bozin *et al.*, 2007).

Sienkiewicz *et al.* (2013) reported that rosemary essential oil contains mainly 1,8-cineole (46.4%), camphor (11.4%) and α-pinene (11.0%). Apart from volatile components, rosemary extracts also include various antioxidant components, primarily categorized under the classes of flavonoids, diterpenoids, and phenolic acids. Del Bano *et al.* (2004) reported the composition of seven flavonoids in rosemary steam, flower, leaves. They investigated the

presence of 7-O-glucoside, hispidulin, diosmin, hesperidin, 30-O-β-D-glucuronide, genkwanin, and isoscutellarein 7-O-glucoside. Other compounds found in rosemary extracts, such as rosmarinic acid and hydroxyhydrocaffeic acid, also have some antioxidant activity.

2.12.2 Antimicrobial activity of rosemary extract

Rosemary extract shows a great antimicrobial activity. Prabuseenivasan *et al.* (2006) confirmed that rosemary essential oil effectively inhibits E. coli ATCC 25922. The minimum inhibitory concentration for rosemary oil against *E. coli* was greater than 6.4 mg/l. Gonelimali *et al.* (2018) studied the effectiveness of rosemary against Gram-positive bacteria (*Bacillus cereus, Staphylococcus aureus*), Gram-negative bacteria (*Escherichia coli, Salmonella enteritidis, Vibrio parahaemolyticus* and *Pseudomonas aeruginosa*), and fungus (*Candida albicans*).

The inhibitory effect of rosemary is the result of the action of rosmarinic acid, rosmaridiphenol, carnosol, epirosmanol, carnosic acid, rosmanol and isorosmanol (Nieto *et al.*, 2018). Vegara *et al.* (2011) reported that the carnosic acid is more efficient against pathogenic bacteria than other extract components, including rosmarinic acid.

Rosemary has been shown to have antimicrobial properties in many dietary tests, including meatballs (Fernández-López *et al.*, 2005), cooked beef (Ahn *et al.*, 2007), and pork sausage (Pandit and Shelef, 1994). Gómez-Estaca *et al.* (2010) reported that rosemary oil inhibits the growth of microorganism that cause food spoilage. Govaris *et al.* (2007) reported an inhibitory effect of dietary supplementation of turkeys with rosemary (5 and 10 g/kg) on the growth of bacteria responsible for spoilage (psychrotrophs, mesophilics, enterobacteria and lactic acid bacteria)

Fernández-López *et al.* (2005) evaluated the antibacterial activity of rosemary extracts in veal meatballs. The results showed a higher antibacterial activity in rosemary extracts, compared to orange and lemon extracts studied. Only rosemary extracts were able to inhibit the 11 bacteria tested (such as *Lb. lactis, Br. thermosphacta, Lb. carnosum, Br. thermosphacta, L. innocua, Lb. sake, Br. thermosphacta, Lc. mesenteroides subsp mesenteroides, L. monocytogenes, Lc. mesenteroides subsp dextranicum, Lb. curvatus*).

2.12.3 Antioxidant activity of rosemary extract

Rosemary extract's antioxidant properties have predominantly been attributed to the phenolic diterpenes, carnosic acid and carnosol, which terminate the cycle of free-radical chain reactions by hydrogen donation and scavenge reactive oxygen species (Basaga *et al.*, 1997).

Aruoma *et al.* (1992) studied the antioxidant and pro-oxidant properties of rosemary. The main constituents with antioxidant properties are carnosic acid and carnosol that are responsible for 90% of the properties. According to Loliger (1991), carnosic acid and carnosol act as potent scavengers of peroxyl radicals. This fact explains the conclusions obtained by Chen *et al.* (1992) , who confirmed that the effect of both compounds on peroxidation of membrane lipids is higher than the effect reported by artificial antioxidants such as BHA, BHT and propyl gallate (Aruoma, 1999).

Sebranek *et al.* (2005) demonstrated that the use of 2500 ppm of a commercial rosemary extract has greater antioxidant capacity when compared to 200 ppm of BHA/BHT when used in refrigerated fresh pork sausage. Lipid oxidation has also been inhibited in pork patties, pork batters and pork sausages through the addition of rosemary extract (Georgantelis *et al.*, 2007; Martínez *et al.*, 2007; Hernández-Hernández *et al.*, 2009).

Production functional yoghurt supplemented with rosemary extract as natural antioxidant has been studied. Antioxidant values were evaluated estimated by DPPH, FRAP, reducing activities and phenolic compound content when fresh and during storage at 5˚C ± 2˚C for 12 days. The modeling results showed that soluble phenolic content in yogurt is increased with increasing the concentration of rosemary extract mixed with skim milk which is strongly correlated with antioxidant activity and decreased over the same time period (Gad and Sayd, 2015).

2.13 Clove (*Syzygium aromaticum*)

Syzygium aromaticum L. commonly known as clove, is an evergreen tree with sanguine flowers belonging to the family Myrtaceae native from the Maluku islands in east Indonesia that grows in tropical climates (Kenney *et al.*, 1992). It grows to a height ranging from 8-12m, having large square leaves. For centuries, the trade of cloves and the search for this highly valued spice stimulated the economic development of this Asian region (Kamatou *et al.*, 2012).

Cloves have long been used in traditional medicine and cuisine, and they produce an essential oil that has been utilized in food flavorings, traditional medicine, and perfume manufacture since ancient (Kenney *et al.*, 1992). Even though cloves are mostly used as a nutritional spice for food in the Western world, they have historically served as a remedy for a variety of health concerns, with the clove anesthetic (due to eugenol), stimulating, antimicrobial, antifungal, antiviral, and antiseptic properties having been known for centuries (Kumar *et al.*, 2012).

Clove is a highly valued spice that has been used for centuries as a food preservative and for a wide range of medicinal purposes (Cortés-Rojas *et al.*, 2014). This versatile chemical is used in a variety of industries, including pharmaceuticals, agriculture, fragrance, flavoring, and cosmetics. Research has shown that it possesses numerous pharmacological effects, including as antibacterial, anti-inflammatory, analgesic, antioxidant, and anticancer properties and insect repellent properties (Kamatou *et al.*, 2012)

The FDA agency has confirmed the safety of clove buds, clove oil, and some clove ingredients as a food supplement (Vijayasteltar *et al.*, 2016), while the WHO has established the acceptable daily uptake of cloves in humans at 2.5 mg/kg body weight (Ogunwande *et al.*, 2005).

2.13.1 Chemical composition of clove

Clove is a rich source of phenolic compounds including flavonoids, hidroxibenzoic acids, hidroxicinamic acids, and hidroxiphenyl propens. Eugenol is the main bioactive compound of clove, which is found in concentrations ranging from 9381.70 to 14650.00 mg per 100 g of fresh plant material (Kamatou *et al.*, 2012). The compounds that are present in greater concentrations are gallic acid and phenolic acids. When compared to other substances, the concentrations of other gallic acid derivatives, such as hidrolizable tannins, are higher (Shan *et al.*, 2005).

Salicylic, elagic, ferulic, and caffeic acids are other phenolic acids present in clove. Clove contains flavonoids such as kaempferol, quercetin, and its glycosilated group, but in lower concentrations. The buds of clove flowers contain concentrations of essential oil of up to 18%. About 89% of clove essential oil is eugenol, with eugenol acetate and β-cariofileno making up the remaining 5% to 15% (Jirovetz *et al.*, 2006).

α-humulen is another significant component that can be detected in clove essential oil at concentrations of up to 2.1%. Clove essential oil also contains other volatile compounds in smaller amounts, such as β-pinene, limonene, farnesol, benzaldehyde, 2-heptanone, and ethyl hexanoate (Khoshdouni, 2017).

2.13.2 Antimicrobial activity of clove extract

The antimicrobial activities of clove have been proved against several bacterial and fungal strains. Ouattara *et al.* (1997) reported that essential oils containing carvacrol and eugenol have the highest antimicrobial properties.

Sofia *et al.* (2007) investigated the antimicrobial activity of various Indian spice plants, including mint, cinnamon, mustard, ginger, garlic, and clove. The 3% aqueous extract of clove demonstrated complete bactericidal effect against all food-borne pathogens tested, including *Escherichia coli, Staphylococcus aureus, and Bacillus cereus*. Clove extract at 1% concentration demonstrated good inhibitory action.

Rajak *et al.* (2011) studied clove oil's antifungal activity against several strains and reported a scale of sensitivity *Mucor sp.>Microsporum gypseum>Fusarium monoliforme > Trichophytum rubrum>Aspergillus sp.>*and *Fusarium oxysporum*. The chromatographic analyses showed that eugenol was the main compound responsible for the antifungal activity due to lysis of the spores and micelles.

Rajkumar and Berwal (2003) used the spice agar method to study the inhibitory impact of clove on *Penicillium chrysogenum, Penicillium verrugosum, Aspergillus flavus, and Aspergillus parasiticus*, with the aim of stabilizing intermediate moisture meat products. The minimum inhibitory concentration of clove at the 80% inhibition level was also estimated. Clove showed minimal inhibitory concentrations of 0.86, 1.12, 1.08, 1.30, and 0.92% (w/v) against *P. chrysogenum, P. verrugosum, Asp. flavus, and Asp. parasiticus*, respectively. At 5% level of clove all spoilage and toxigenic mold growth was inhibited during 25 days of study period.

Mabrouk and El-Shayeb (1980) studied the effects of black pepper, cinnamon, peppermint, cumin, ginger, and clove on growth and aflatoxin production of *Aspergillus flavus* on rice powder corn steep medium. The first five spices were evaluated for their ability

to suppress aflatoxin synthesis, rather than mycelial development. Clove decreased mycelial growth and aflatoxin formation at concentrations above 0.1%.

2.13.3 Antioxidant activity of clove extract

Shan *et al.* (2005) used high performance liquid chromatography to identify and quantify the main phenolic compounds found in 26 spices. The ABTS technique was then used to analyze the in vitro antioxidant activity of the compounds. Results showed a strong relationship between antioxidant activity and polyphenol concentration. The spice with the highest polyphenol content and antioxidant activity was clove (buds). Phenolic acids (gallic acid), flavanol glucosides, phenolic volatile oils (eugenol, acetyl eugenol), and tannins were the major types of phenolic compounds that were identified. Clove has shown great potential as a radical scavenger and commercial source of polyphenols.

Antioxidants are known to reduce lipid oxidation through free radical scavenging. Gülçin *et al.* (2012) found cloves to be more effective at scavenging free radicals than chemical antioxidants such as BHA, BHT, α-tocopherol, and Trolox. The results of the DPPH assay indicated that 45 µg/ml of clove oil showed 83.6% inhibition. With an increase in concentration, clove oil's capacity to scavenge free radicals (DPPH) also increased.

Clove and lavender ethanol and aqueous extracts at 20, 40, and 60 µg/ml showed up to 95% inhibitions in metal quelants, superoxide radical capture, and DPPH radical scavenging assays. According to Cortés-Rojas *et al.* (2014) the powerful antioxidant activity of both extracts can be attributed to their strong potential to donate hydrogen, chelate metal, and scavenge free radicals, hydrogen peroxide, and superoxide.

2.14 Herbs and spices incorporated in cheeses

Herbs and spices are used in cheese manufacture as flavorful, antioxidant, and antimicrobial ingredients, as well as to increase the stability and usefulness of cheese. These cheeses regularly considered as specialty cheeses. Typically, these cheeses are regarded as specialty cheeses (Ritota and Manzi, 2020). Some common herbs and spices used in cheesemaking are presented in Table 2.6

Table 2.6 Common herbs and spices used in cheesemaking.

Herbs/Spices	Botanical name	Examples of cheese with herbs/spices
Chilli	*Capsicum frutescens var. chili*	Cheddar, Monterey Jack
Red pepper	*Capsicum frutescens var. faseiculatum*	Cheddar, Monterey Jack
Green pepper	*Capsicum frutescens var. grosum*	Monterey Jack, processed cheese
Paprika	*Capsicum frutescens var. tetragonium*	Bouletted'Avesnes
Black pepper	*Piper nigrum*	Gouda
Mustard seeds	*Brassica sinapis alba*	Monterey Jack
Caraway seeds	*Carumcarvi*	Gouda, Liptauer
Cumin	*Cuminun cyminum*	Gouda, Liptauer
Garlic	*Allium sativum*	Cheddar, Monterey Jack
Dill	*Anethum graveolens*	Processed cheese products
Basil	*Ocimum basilicum*	Feta, Cheddar, Monterey Jack
Fenugreek	*Trigonella foenum graecum*	Gouda
Tarragon	*Artemisia dracunculus*	Bouletted'Avesnes
Sage	*Salvia officinalis*	Cheddar
Chives	*Allium schoenoprasum*	Cheddar

Source: Hayaloglu and Fox (2008)

Part III

Materials and methods

3.1 Materials

3.1.1 Sample

Raw cow milk of local breed was collected from the local market of Dharan. Rosemary and clove were purchased from the local market of Dharan. Rennet was collected from the Kamdhenu Dairy Development Cooperative (KDDC), Itahari.

3.1.2 Packaging material

Low density polyethylene (LDPE) bags used for packaging of samples were purchased from Kamdhenu Dairy Development Cooperative (KDDC), Itahari.

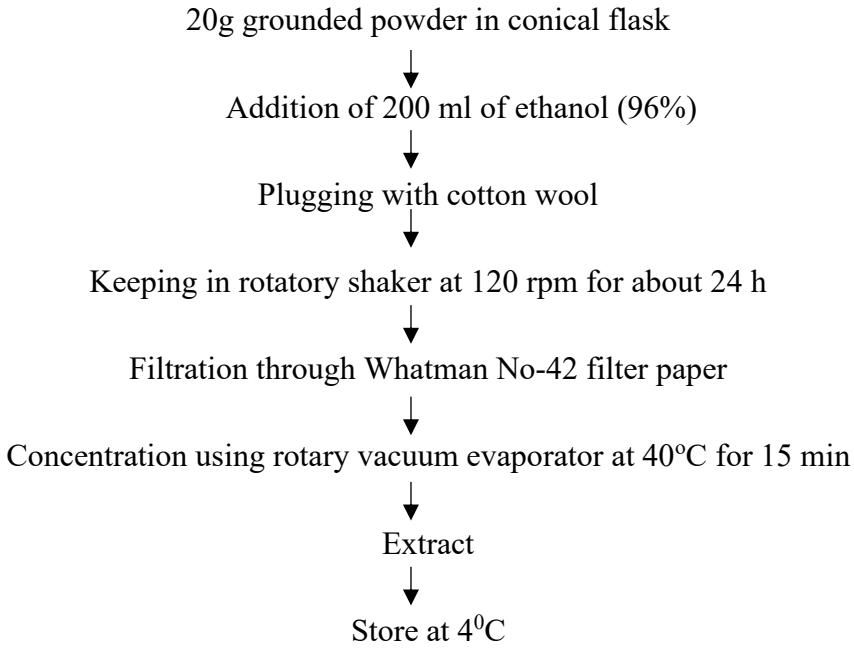
3.1.3 Chemicals and apparatus

All the chemicals, glass wares and equipment required were used from the lab of Central Campus of Technology (CCT), Dharan.

3.2 Methods

3.2.1 Preparation of extract

The rosemary and clove extracts were prepared individually according to the method followed by Yadav *et al.* (2015) with slight modifications. Rosemary leaves and clove buds were cleaned and dried separately in cabinet dryer at 50^{o}C for 4-5 h and ground in the grinder to a powder form separately. 20g grounded powder was taken separately in a conical flask and 96% ethanol was added, maintaining 1:10 (w/v) ratio. The flask was plugged with cotton wool and kept in rotatory shaker at 120 rpm for about 24 h. The filtrate was filtered through Whatman No-42 filter paper and concentrated using rotary vacuum evaporator 27 (IKA HB 10 digital) at 40^{o}C for about 15 min. The extract obtained was stored at 4^{o}C in a conical flask with screw cap and covered with aluminum foil until usage separately.



Source: Yadav *et al.* (2015)

Fig.3.1 Preparation of extracts

3.2.2 Analysis of extract

3.2.2.1 Determination of extraction yield

The extracts were filtered using Whatman No. 42 filter paper, the filtered extracts were concentrated by a rotary evaporator 27 (IKA HB 10 digital), and the residual extracts were dried. The percentage yield was obtained using dry weight (Adam *et al.*, 2019).

$$\% \text{ Extraction yield} = \frac{\text{Weight of extract residue after solvent removal (g)}}{\text{Total weight of dried powder (g)}} \times 100$$

3.2.2.2 Determination of total phenolic content of extract

Total phenolic content (TPC) of extracts was determined by Folin and Ciocalteu (FC) colorimetric method described by Kupina *et al.* (2019). In a test tube, 7.5 ml of distilled water was added, then 0.5 ml of FC reagent and 0.5 ml of extract was added to it. The mixture was mixed well and allowed to rest for 6 min. Then, 1.5ml of 10% solution of 20% sodium

carbonate was added and mixed well. The mixture was allowed to stand for 2 h at 30°C in incubator before the absorbance measured at 765 nm in UV-visible spectrophotometer. A mixture of reagent without the sample was used as a blank. The total phenolic content of the extracts was determined using a calibration curve (Fig. D.1 in Appendix D) obtained from gallic acid solutions ranging from 40 mg/l to 400 mg/l. The total phenol content was then calculated based on the equation derived from the calibration curve and expressed as mg GAE/g on dry basis.

3.2.2.3 Determination of total flavonoid content

Total flavonoid contents (TFC) in the extracts were determined by aluminum chloride colorimetric assay as described by Phuyal *et al.* (2020). Stock solution (4 mg/ml) of quercetin was prepared by dissolving 4 mg of quercetin in 1 ml of ethanol. This standard solution was diluted serially to make various concentrations of 0.25 mg/ml, 0.5 mg/ml, 0.75 mg/ml, and 1 mg/ml solutions. 1 ml quercetin of each concentration was added to the test tube containing 4 ml of distilled water. At the same time, 0.3 ml of 5% $NaNO_2$ was added to the test tube and 0.3 ml of 10% $AlCl_3$ after 5 min. Then, 2 ml of 1 M NaOH was added to the mixture after 6 min and volume of the mixture was leveled to 10 ml by adding 4.4 ml of distilled water.

Similar procedure as described for quercetin was followed for the extract also, and the absorbance was measured by spectrophotometer at 510 nm. Readings were taken in triplicate, and the average value of absorbance was used to calculate the total flavonoid content. The flavonoid content was expressed as quercetin equivalent (mg QE/g) using the linear equation based on the standard calibration curve (Fig D.2 in Appendix D).

3.2.2.4 Determination of antioxidant activity

Antioxidant activity (AA) of the extracts was tested by DPPH radical scavenging assay as described by Gulcin and Alwasel (2023) with slight modification. 0.1 mM DPPH solution was prepared by dissolving 4 mg of DPPH in 100 ml of methanol. 1 ml of the sample extract was taken in a test tube, and 4 ml of freshly prepared methanolic solution of DPPH was added. The mixture was shaken well then left for 30 minutes in the dark. Subsequently, the absorbance was measured at 517 nm using a UV-vis spectrophotometer. A parallel control was run using methanol instead of the sample.

The radical scavenging activity is expressed as the radical scavenging percentage using the following equation:

$$\text{Inhibition } \% = \frac{\text{Ao-As}}{\text{Ao}} \times 100$$

Where, Ao = average absorbance of the control (DPPH) solution

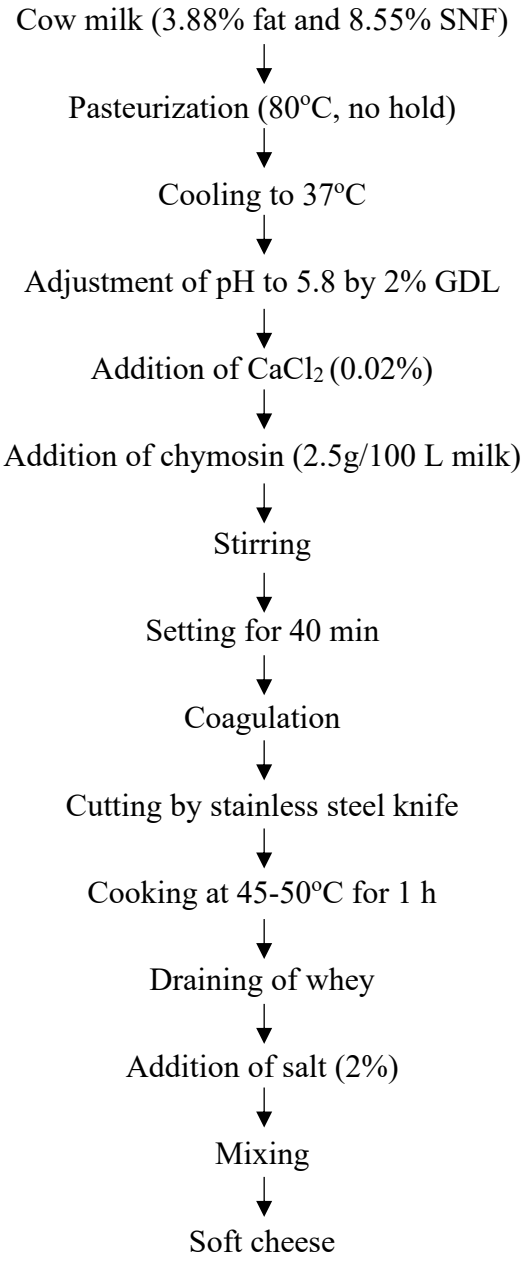
As = average absorbance of the sample

3.3 Physiochemical analysis of milk

Physiochemical parameters such as pH, titratable acidity, fat content, protein content, moisture, solid not fat were analyzed by methods given by AOAC (2005).

3.4 Preparation of soft cheese

Cheese was manufactured according to the method given by Zheng *et al.* (2021) with slight modifications is shown in Fig.3.2. Milk having 3.88% fat and 8.55% SNF was heated to 80ºC and left for cooling. When the milk temperature decreased to 37ºC, the pH was adjusted to 5.8 by using 2% glucono-delta lactone (GDL) and $CaCl_2$ was added at the rate of 0.02%. Rennet at the rate 2.5g/100 L of milk was added when the milk pH was 5.8 at 37ºC. The cheese milk was gently stirred and allowed to coagulate for 40 min. The curd was then cut vertically and horizontally by stainless steel knife and it was further cooked at 45- 50ºC for 1 h until the curd became firm. Whey was drained using cheese cloth and curd was pressed for 10 min to release the whey. 2% dry salt was added into the curd and mixed properly.



Source: Zheng *et al.* (2021)

Fig.3.2 Preparation of soft cheese

3.4.1 Extract incorporation in cheese

The cheese curd was divided into 11 portions. The first portion was control. Rosemary extract and clove extract was incorporated to soft cheese separately at concentrations of 0.1%, 0.2%, 0.3%, 0.4%, 0.5%. Then, the curd was worked so as to distribute the extract as uniformly as possible. Then, the prepared cheeses were vacuum packed in low density

polyethylene (LDPE) and stored in refrigerator at below 5ºC and the shelf life was assessed at 7 days intervals over a storage period of 28 days.

3.5 Yield of cheese

The yield of cheese samples was calculated using the equation as per Dehkordi *et al.* (2022)

$$\text{Cheese yield (\%)} = \frac{\text{Weight of cheese}}{\text{Weight of milk}} \times 100$$

3.6 Physicochemical analysis of cheese during storage

The prepared cheeses were analyzed for physicochemical composition at interval of 7 days for 28 days.

- Moisture: The moisture content of cheese samples was estimated by oven-drying method as described in AOAC (2005).
- pH: The pH of cheeses was determined by the method mentioned in AOAC (2005).
- Protein: The protein content of milk and cheese samples was estimated by Kjeldahl method as mentioned in AOAC (2005).
- Fat: Fat content cheese samples was estimated by the method in AOAC (2005).
- Ash: Ash content of cheese samples was estimated by dry ashing method as mentioned in AOAC (2005).
- Acidity: Acidity of cheese samples was determined by the method as mentioned in AOAC (2005).

3.7 Determination of total free amino acid of cheeses during storage

The level of total free amino acid (FAA) was determined by the method of Folkertsma and Fox (1992). A 100µl of water-soluble nitrogen was diluted with H_2O into 1 ml in which 2ml of Cd-ninhydrin reagent. The reagent was prepared by dissolving 0.8 g ninhydrin in a mixture of 80 ml ethanol and 10 ml glacial acetic acid followed by the addition of 1 g cadmium chloride ($CdCl_2$) dissolved in 1 ml of distilled water. Then, the tubes were vortexed and heated at 84°C for 5 min and cooled in ice water. The absorbance was determined at 507 nm. Thus, obtained results was expressed as mg leucine per g cheese by reference to a

standard curve which was first be prepared using leucine at various concentrations (0.05 – 0.50 mg leu/ml).

3.8 Determination of free fatty acid of cheeses during storage

Lipolysis was measured as acid degree value (ADV) according to the procedure of Nunez *et al.* (1986) and Hayaloglu (2007). A 10 g sample of cheese was macerated in a mortar with 6 g of anhydrous sodium sulfate (Na_2SO_4), and then 60 ml of diethyl ether was added to the mixture in a 100 ml screw-capped bottle. After 2 h of stirring, the mixture was filtered using Whatman No. 1 paper. Following three consecutive times of re-suspending the precipitate in a bottle with 20 ml diethyl ether, filtration was performed. Then, diethyl ether was removed from the filtrate at 50°C using a rotary evaporator. After complete removal of the diethyl ether, the liquid fat was transferred into a flask and weighed. A 5 ml of ethanol and diethyl ether (1:1) were added to the flask. The total mixture was titrated with 0.05 N alcoholic potassium hydroxide (KOH) solution. The result was expressed as mg KOH per g of cheese.

3.9 Microbiological analysis of cheeses during storage

The microbiological analysis of cheeses was performed as per the method described by AOAC (2005). The total viable count was determined by plate count agar (PCA), yeast and molds by potato dextrose agar (PDA) and coliform bacterial count by using violet red bile agar (VRBA).

3.10 Sensory evaluation of cheese during storage

Control cheese and the extract incorporated cheese samples were evaluated organoleptically on the 1st, 7th, 14th, 21st and 28th day of ripening by 10 semi-trained panelists following the recommendations of IDF (1992). The panelists were screened initially based on their sensitivity to recognize basic tastes. Training session was then conducted to define sensory terminologies. Test samples were served as descriptive stimuli for the language development and sensory descriptors for appearance, texture, taste, odor, aftertaste and overall acceptability were developed. The cheese samples were then presented in a properly ventilated and well-lit laboratory and the panelists evaluated each sample for every attribute using a five-point hedonic scale, with 1 being poor and 5 being the excellent quality. The specimen of sensory evaluation card is shown in Appendix A.

3.11 Statistical Analysis

Data were analyzed using analysis of variance (ANOVA) at 95% confident level using IBM SPSS Statistics (Version 27). The graph and general calculations were done in Microsoft Excel (2019). Means of the data were compared by using Tukey's honestly significant difference (HSD) method at 5% level of significance.

Part IV

Results and discussion

In this study, the extracts of rosemary and cloves were incorporated individually in the cheeses at varying concentrations (0.1%, 0.2%,0.3%, 0.4% and o.5%) and the best was selected through sensory analysis. The cheeses were analyzed for its physicochemical, microbiological and sensory characteristics during 28 days of storage period at interval of 7 days.

4.1 Extraction yield

The yield of rosemary extract was found to be 18.89%, which is slightly higher than the findings of Chaqroune and Taleb (2022) who reported 17.32% . The variation in the extraction yield of rosemary could be due to a combine factors related to variation in rosemary species and varieties, extraction process and harvesting time and storage condition (Gu *et al.*, 2021).

The yield of clove extract was found to be 36.45% which is lower than the findings of Ishaq *et al.* (2019) who reported 41.47%. Jayaprakasha *et al.* (2001) reported that variation in the extraction yields of different extracts is attributed to difference in polarity of compounds found in plants.

4.2 Phytochemical analysis of extracts

The phytochemical analysis of rosemary and clove extracts is presented in Table 4.1.

Table 4.1 Phytochemical analysis of rosemary and clove extracts.

Phytochemicals	Rosemary extract	Clove extract
Total phenolic content (TPC) (mg GAE/g)	36.11±0.88	52.88±1.277
Total flavonoid content (TFC) (mg quercetin/g)	8.02±2.59	13.56±0.91
Antioxidant activity (% inhibition)	68.51±0.68	75.14±2.15

Note: Values are the means ± standard deviation of triplicate analysis.

The TPC of rosemary extract was found to be 36.11±0.88mg GAE/g dry matter. Zhang *et al.* (2016) reported the content of TPC in rosemary extract was 34.18±1.22 mg GAE/g dry matter which was somewhat similar to the above findings. Zeroual *et al.* (2022) stated that the TPC of the rosemary extract lies in between 12.48±1.17-34.72±1.65mg GAE/g dry matter. The TPC content of rosemary extract was found to be 52.88±1.27mg GAE/g dry matter. Calderón-Oliver and Ponce-Alquicira (2021) reported the content of TPC in clove extract was 54.3±7.3 mg GAE/g dry matter which was similar to the obtained value. Several studies reported that the differences in polyphenol content could be attributable to biological factors (genotype, cultivars), as well as environmental (temperature, light) conditions. Moreover, the extraction of phenolic compounds depends on the type of solvent used, the degree of polymerization of phenolics, and their interaction (Mamun *et al.*, 2016).

The flavonoid content of rosemary extract was found to be 8.02±2.59 which slightly lower than the findings of Bianchin *et al.* (2020). The result was similar to Nadia and Rachid (2016). The flavonoid content of clove extract was found to be 13.56±0.91 mg QE/g of extract which is similar to the findings of Indiarto *et al.* (2024) i.e. 14.54±0.31 mg QE/g extract. The differences in the flavonoid contents may be due to the variation in species, method of analysis, harvesting time and climatic conditions of growing area (Bibi *et al.*, 2022).

The antioxidant activity of rosemary extract was found to be 68.51% inhibition, lower than the findings of Santos *et al.* (2012) who reported a 90.14%. Xu *et al.* (2023) reported that the antioxidant activity of clove extract to be 95.28 which is higher than above findings. The difference in the results may be due to the variation in species and method of analysis (Manganaris *et al.*, 2018).

4.3 Physicochemical properties of milk

Table 4.2 The proximate composition of cow milk is given in Table 4.2.

Parameters	Cow milk
Moisture (%)	87.25±0.13
Fat (%)	3.88±0.02
Protein (%)	3.25±0.04
Ash (%)	0.77±0.01
SNF (%)	8.55±0.05
Acidity (as % lactic acid)	0.14±0.01
pH	6.65± 0.05

Note: Values are the mean ± standard deviation of three determinations.

The result presented in Table 4.2 revealed the moisture, fat, protein, ash, SNF, acidity and pH in cow milk whose values are similar to those reported by Ismael and Hadi (2024) and any variation may be due to difference in cow breed, feeding, milking condition and milking time.

4.4 Sensory evaluation of rosemary extract incorporated soft cheese

Soft cheese without incorporation of rosemary extract was considered as control and denoted as A. Rosemary extract was incorporated in the cheese at the concentration of 0.1%, 0.2%, 0.3%, 0.4% and 0.5% were denoted as B, C, D, E and F respectively. The sensory evaluation was carried by using a 5-point hedonic scale. Fig. 4.1 shows the mean sensory score of rosemary extract incorporated soft cheese.

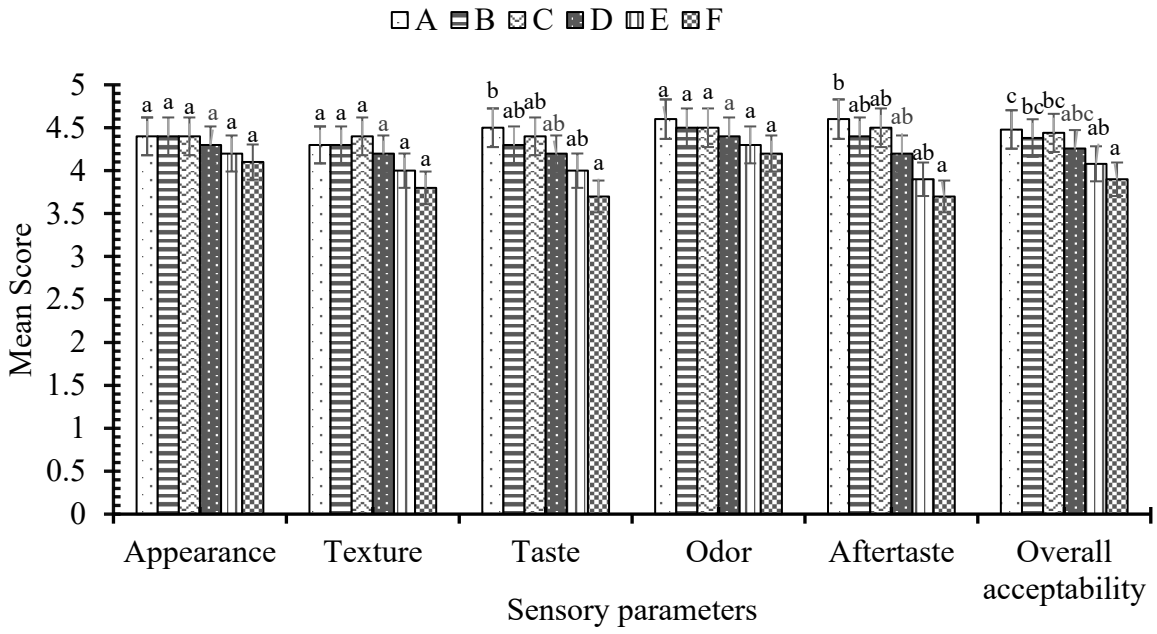


Fig. 4.1. Mean sensory score of rosemary extract incorporated soft cheeses

Note: Values on top of the bars bearing similar superscript are not significantly different at 5% level of significance. Vertical error bars represent the standard deviation of scores given by panelists.

4.4.1 Appearance

The statistical analysis showed that the mean appearance score of cheese samples was not significantly ($P>0.05$) different, though a slight decline was noted at higher concentrations. The appearance of the control cheese was white, whereas cheese with the rosemary extract ranged from fainty green to green with the increasing concentration. Similar result was observed by Salih *et al.* (2020) who studied the effect of *Moringa oleifera* seeds extract on the sensory characteristics of white cheese.

4.4.2 Texture

The statistical analysis showed that incorporation of rosemary extract in cheeses had no significant effect ($P< 0.05$) on texture. However, the mean texture score of cheese C was higher than that of other cheese samples. The mean score for the texture decreased with the increase in the concentration of the rosemary extracts in the cheese. Fernandes *et al.* (2016) reported that that incorporating rosemary essential oil at optimal levels can improve certain

quality factors; however, exceeding these levels may lead to adverse effects on texture resulting into more brittle texture.

4.4.3 Taste

The statistical analysis showed that the incorporation of rosemary extract in cheese had significant effect ($P< 0.05$) on flavor. Post hoc analysis showed that cheese A was not significantly different ($P>0.05$) from cheeses B, C, D, E; though the mean taste score of cheese C was higher than other rosemary extracts incorporated cheeses. Cheese A was significant different ($P<0.05$) from sample F. At the lower concentration, the taste remains well-balanced and imparts a light herbal note that enhance the taste without overpowering the cheese's inherent taste (Hala *et al.*, 2010). However, as the concentration of the extract in the cheese increases, it can lead to bitterness or an overly strong herbal flavor that might not be appealing for all consumers (Himed-Idir *et al.*, 2021).

4.4.4 Odor

The statistical analysis showed that the mean odor scores of cheese samples were not significant different ($P> 0.05$), however there was a slight decline in the mean odor scores with the increase in concentrations of extract. High concentrations of rosemary extract contain higher levels of phenolic components such carnosic acid and rosmarinic acid. These compounds may react with the cheese matrix, potentially resulting in the reduction of volatile aromatic compounds that contribute to rosemary's distinctive odor that resulted into lower sensory score (Ghasemian *et al.*, 2024).

4.4.5 Aftertaste

The statistical analysis showed that incorporation of rosemary extract in cheese had significant effect ($P< 0.05$) on aftertaste. From post hoc analysis, the mean sensory score for aftertaste of cheese A was the highest of all the cheese samples. Cheese A was not significantly different ($P>0.05$) from cheese B, C, D and E. Cheese F showed lowest mean score and was significantly different ($P<0.05$) from cheese A. Higher concentrations of rosemary extracts are known to impart bitter and astringent flavors, which can overshadow the desirable aftertaste of the cheese that could lead to less favorable sensory attributes (Ghasemian *et al.*, 2024).

4.4.6 Overall Acceptability

The statistical analysis showed that the mean overall acceptability scores of samples were significantly different ($P< 0.05$). The post hoc analysis showed that cheese A scored highest score and was significantly different ($P<0.05$) from cheese F, which showed lowest score in overall acceptability which may be due to increase in concentration of extract. Cheese B and C were not significantly different ($P>0.05$) from cheese A, D and E. Similarly, cheese F did not significantly differ from cheese D and E. However, cheese C achieved high mean overall acceptability score and was accepted as the best sample by panelist among the extract treated cheese samples.

4.5 Sensory evaluation of clove extract incorporated soft cheese

Soft cheese without incorporation of clove extract was considered as control and denoted as A. Clove extract added in cheese at the concentration of 0.1%, 0.2%, 0.3%, 0.4% and 0.5% were denoted as B', C', D', E' and F' respectively. The sensory evaluation was carried by using a 5-point hedonic scale. Fig. 4.2 shows the mean sensory score of clove extract incorporated soft cheese.

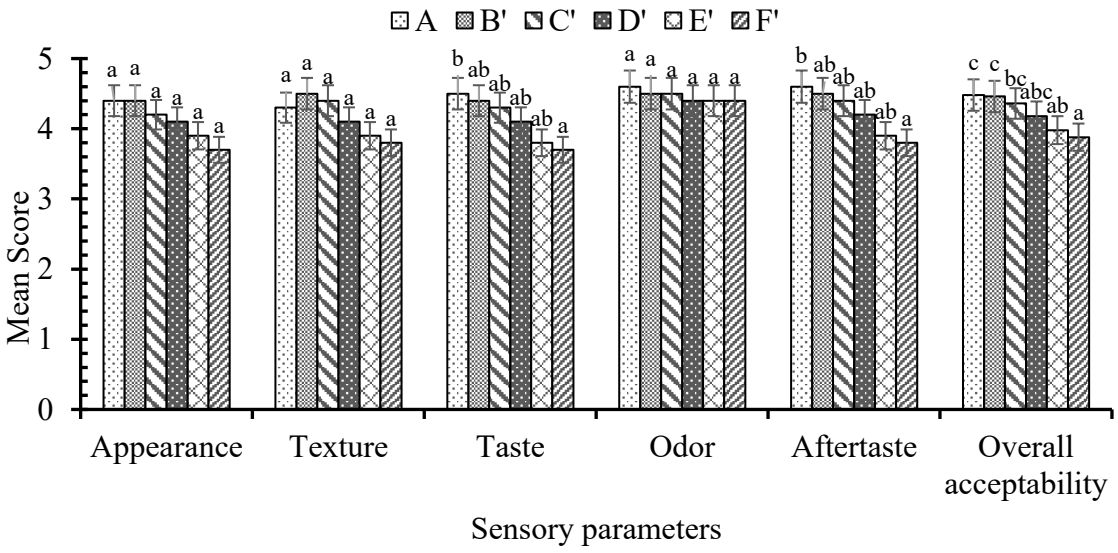


Fig. 4.2 Mean sensory score of clove extract incorporated soft cheeses

Note: Values on top of the bars bearing similar superscript are not significantly different at 5% level of significance. Vertical error bars represent the standard deviation of scores given by panelists.

4.5.1 Appearance

The ANOVA showed that the mean appearance scores of cheese samples were not significantly different (P>0.05). However, the mean score for appearance was found to be slightly decreasing with the increase in concentration of extracts incorporated to soft cheese which may be due to the phenolic compound present in clove that impart a deeper brownish in higher concentration hue to cheese, leading to lower appearance score (Zhang *et al.*, 2017).

4.5.2 Texture

The ANOVA revealed that incorporation of clove extract in cheese had no significant effect (P>0.05) on texture, though cheese B' had the highest score among all the samples whereas as the concentration of extract increased the texture of cheese becomes softer. This may be due to the interaction between clove oil components and milk proteins that may affect the protein network formation during cheese production. Such interactions can interfere with casein micelle aggregation and curd formation, resulting in a softer cheese texture Khani and Nouri (2024).

4.5.3 Taste

The ANOVA showed that incorporation of clove extract in cheeses had significant effect (P< 0.05) on taste. Cheese A had the highest mean score among all cheese samples. Sample B', C', D' and E' were not significantly different (P>0.05) from cheese A. Sample F' showed a significant difference from cheese A. Clove oil is rich in eugenol, a compound known for its strong aromatic properties, which imparts a distinctive spicy and pungent taste to the cheese. As the concentration of clove extract increases, these sensory characteristics become more pronounced, potentially enhancing the cheese's taste complexity which result into lower sensory acceptability (Charfi *et al.*, 2021).

4.5.4 Odor

The ANOVA revealed that the mean odor score of cheese samples was not significantly different (P>0.05), though a slight decrease in mean odor score was observed with increase in concentration of extract. As the concentration of clove extract in cheese increases, spicy

and pungent aroma becomes more pronounced which potentially affect the consumer acceptance (Charfi *et al.*, 2021).

4.5.5 Aftertaste

The ANOVA showed that incorporation of clove extract in cheese had significant effect ($P<0.05$) on aftertaste. The mean sensory score for aftertaste of sample A was found to be the highest among all the cheese samples. Sample B', C', D' and E' was not significantly different ($P>0.05$) from sample A. The score for the sample C', D', and E' were found to be slightly lower than sample A and B'. Sample F' was significantly different ($P<0.05$) from cheese A and had lowest score in terms of aftertaste. Chon *et al.* (2020) reported that as the concentration of clove extract increases, characteristic flavor becomes more pronounced, which can lead to a sustained aftertaste in the cheese that could be perceived as overpowering by consumers.

4.5.6 Overall acceptability

The ANOVA showed that incorporation of clove extract in cheese had significant effect ($P< 0.05$) on overall acceptability of samples. Post hoc analysis showed that sample A received the highest score and was not significantly different from sample B'. Sample F' had lowest score in overall acceptability which could be result of higher concentration of extract incorporated in it. Therefore, sample B' with lowest concentration of clove extract was considered as the best cheese among the clove extract incorporated cheese samples in terms of overall acceptance.

4.6 Yield of cheese

The yield of cheese was found to be 15.10% which is slightly lower than yield obtained by Czyżak-Runowska *et al.* (2020). The yield can vary depending up factors like milk composition, types of rennet used and the cheese making process (Cipolat-Gotet *et al.*, 2013).

4.7 Physicochemical analysis of soft cheeses during storage period

The changes in physicochemical properties of soft cheeses incorporated with extracts throughout 28 days of storage are shown in the Table 4.3.

Table 4.3 Physicochemical changes occurring in soft cheeses during storage.

Parameters	Storage days	Treatments		
		Control cheese	Rosemary extract treated cheese	Clove extract treated cheese
Moisture (%)	1	58.48±0.06[a]	58.55±0.15[a]	58.47±0.32[a]
	7	57.18±0.48[a]	57.68±0.61[a]	57.55±0.06[a]
	14	56.84±0.14[a]	56.98±0.15[a]	57.17±0.22[a]
	21	55.71±0.74[a]	56.10±0.31[a]	56.75±0.03[a]
	28	54.81±0.25[a]	55.76±0.40[b]	55.96±0.46[b]
Fat (db) (%)	1	42.58±0.02[b]	42.23±0.01[a]	42.19±0.02 [a]
	7	43.60±0.01[c]	42.61±0.02[b]	42.69±0.01[a]
	14	44.58±0.02[c]	43.34±0.01[b]	43.21±0.01[a]
	21	45.26±0.01[c]	44.69±0.01[b]	44.37±0.02[a]
	28	46.19±0.02[c]	45.74±0.02[b]	45.40±0.01[a]
Protein (db) (%)	1	31.84±0.05[a]	31.76±0.04[a]	31.80±0.02[a]
	7	31.88±0.02[a]	31.83±0.05[a]	31.85±0.02[a]
	14	31.94±0.03[a]	31.92±0.01[a]	31.93±0.02[a]
	21	32.02±0.02[b]	31.99±0.02[a]	31.97±0.01[a]
	28	32.15±0.01[a]	32.13±0.01[a]	32.12±0.01[a]
Ash (db) (%)	1	7.43±0.07[a]	7.24±0.04[a]	7.32±0.05[a]
	7	7.62±0.03[b]	7.37±0.06[a]	7.41±0.06[a]

	14	7.72±0.04[b]	7.51±0.03[a]	7.55±0.05[a]
	21	7.80±0.03[b]	7.65±0.05[a]	7.69±0.03[a]
	28	7.89±0.02[b]	7.69±0.01[a]	7.75±0.05[a]
pH	1	5.79±0.01[a]	5.83±0.005[a]	5.86±0.01[a]
	7	5.69±0.05[a]	5.77±0.02[a]	5.79±0.01[a]
	14	5.53±0.02[a]	5.69±0.01[b]	5.72±0.01[b]
	21	5.41±0.05[a]	5.60±0.01[b]	5.62±0.01[b]
	28	5.30±0.02[a]	5.51±0.01[b]	5.52±0.01[b]
Acidity (%)	1	0.14±0.02[a]	0.12±0.01[a]	0.13±0.01[a]
	7	0.16±0.01[a]	0.13±0.02[a]	0.14±0.01[a]
	14	0.20±0.02[b]	0.15±0.01[a]	0.15±0.01[a]
	21	0.22±0.01[b]	0.17±0.01[a]	0.18±0.02[a]
	28	0.24±0.01[b]	0.19±0.01[a]	0.20±0.01[a]

Note. Values are means ± standard deviation of three determinations. Values in rows having different superscript are significant different at 5% level of significance.

The treatment with rosemary and clove extract did not significantly influence ($P>0.05$) the moisture content of soft cheese samples up to 21 days of storage. However, after 21 days, soft cheese incorporated with rosemary and clove extracts showed significant ($P<0.05$) higher moisture contents compared to control which The moisture contents of all cheese showed a decreasing trend which is attributed to both inherent tendency to de-whey and the effect of drying environment condition (Sanjuán *et al.*, 2002). Similar result was obtained by Hamad and El-Kadi (2020).

The protein content of cheese treatments was increased with the progress of storage periods, and observed no significant difference ($P>0.05$) in protein content among cheese treatments, including the control cheese, demonstrating that incorporation of rosemary and clove extracts to soft cheese did not influence protein content during storage. However, protein content increased gradually during refrigerated storage (4±1°C) which may be due

to the decrease in moisture content that increases the amount of whey released as storage periods progress (Abdel-Latif *et al.*, 2021). The result is in accordance with Hassanien *et al.* (2014) and Abd Elmontaleb *et al.* (2020).

As the storage period proceeds, the fat content of control as well as extract incorporated cheeses showed an increasing trend mainly because of decrease in moisture content (Ratiba *et al.*, 2006). There was significant difference ($P < 0.05$) in fat content among cheese samples throughout the storage days. These results coincided with those reported by Khalaf and Ali (2023).

The ash content in cheese consists of salt, typically added to the curd, along with mineral compounds derived from sodium, potassium, calcium, and magnesium chlorides, as well as phosphates and citrates found in milk salts (Guinee and Fox, 2017). The ash content of all the cheese sample increased with the progress of storage days with the significance difference ($P<0.05$) on the 7 days of storage between extract treated samples and control samples. The control cheese treatments showed the highest content of ash compared to other rosemary and clove extract incorporated cheeses. Similar results were obtained by Khalaf and Ali (2023).

The pH level and acidity are crucial factor in cheese quality, influencing its texture, flavor, and shelf life. The pH changes in cheese showed a trend opposite to that of total acidity. Up to 7 days there were no significance difference ($P>0.05$) in both pH and acidity of all cheese treatments but after that pH value decreased significantly ($P<0.05$) whereas acidity increased significantly ($P<0.05$) in the control and the cheese treated with extracts with the progress of the storage period. Control cheese had the lowest pH whereas cheese incorporated with clove extract had the highest pH during the storage. Similarly, development of acidity was slightly slower in the cheese treated with extract. Similar trend was reported by Shan *et al.* (2011) and Ismael and Hadi (2024). This decrease in pH and increase in acidity is likely due to the production of lactic acid by lactic acid bacteria during storage (El-Sayed and Shazly, 2024).

4.8 Changes in free amino acid during storage

The proteolytic activity has a direct effect on the concentration of peptides and free amino acids in cheese (Shori and Baba, 2015). Free amino acids are precursors of several volatile aroma compounds found in cheese, including amines, carboxylic acids, thiols, esters, alcohols, aldehydes, and thioesters (Mei *et al.*, 2015). Certain free fatty acids play crucial roles in flavor development. Proline and serine are associated with sweetness, while arginine is associated with bitterness (Izco and Torre, 2000).

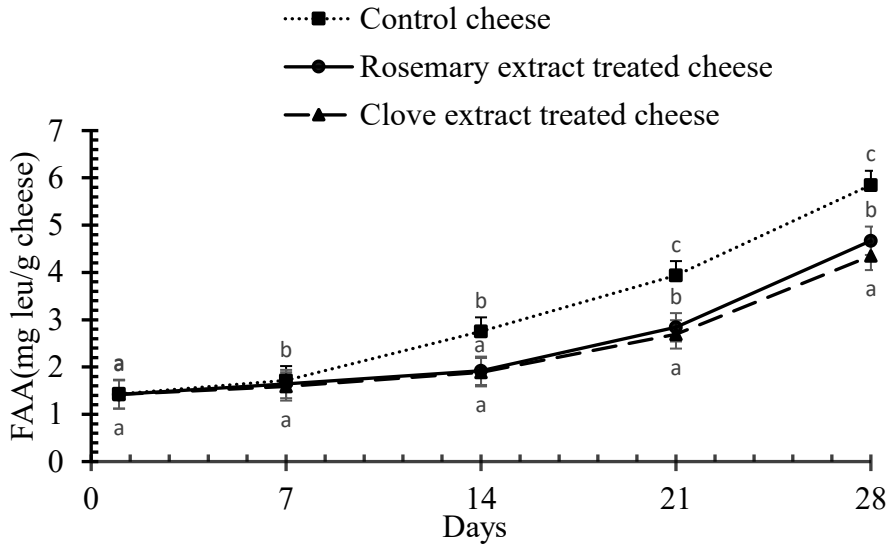


Fig.4.3 Free amino acid of control and extract treated cheeses throughout 28 days of ripening.

Note: Data represents mean ± standard deviation different superscript are significant different at 5% level of significance.

Fig.4.3 shows the level of free amino acid (FAA) produced in control and extract incorporated cheeses throughout the 28 days of storage period. The addition of extracts in cheese had a significant ($P<0.05$) effect in free amino acid content of cheese during storage for 28 days. An increasing trend as storage period progress was observed for control and extracts incorporated cheese with a value significantly ($P<0.05$) higher for control cheese after 7 days of storage period. There was no significant ($P>0.05$) difference in free fatty acid between clove and rosemary extract incorporated cheese up to 14 days. The control samples showed the highest values of FAA whereas clove incorporated cheese had the lowest values in comparison to rosemary extract incorporated cheese and control during 28 days of storage.

Similar observation was reported by Shori *et al.* (2022) who found that addition of Star anise (*Illicium verum)*, Guava (*Psidium guajava)* and Turmeric (*Curcuma longa)* had low FAA in cheese compared to control during 28 days of ripening.

4.9 Changes in free fatty acid during storage

Lipolytic activity in cheese is mainly due to lipase enzyme that can be derived from milk, from microorganisms and possibly from rennet (Tarakci and Temiz, 2009). Acid degree value (ADV) is used to characterize the degree of lipolysis in cheese (Litopoulou-Tzanetaki and Tzanetakis, 2011).

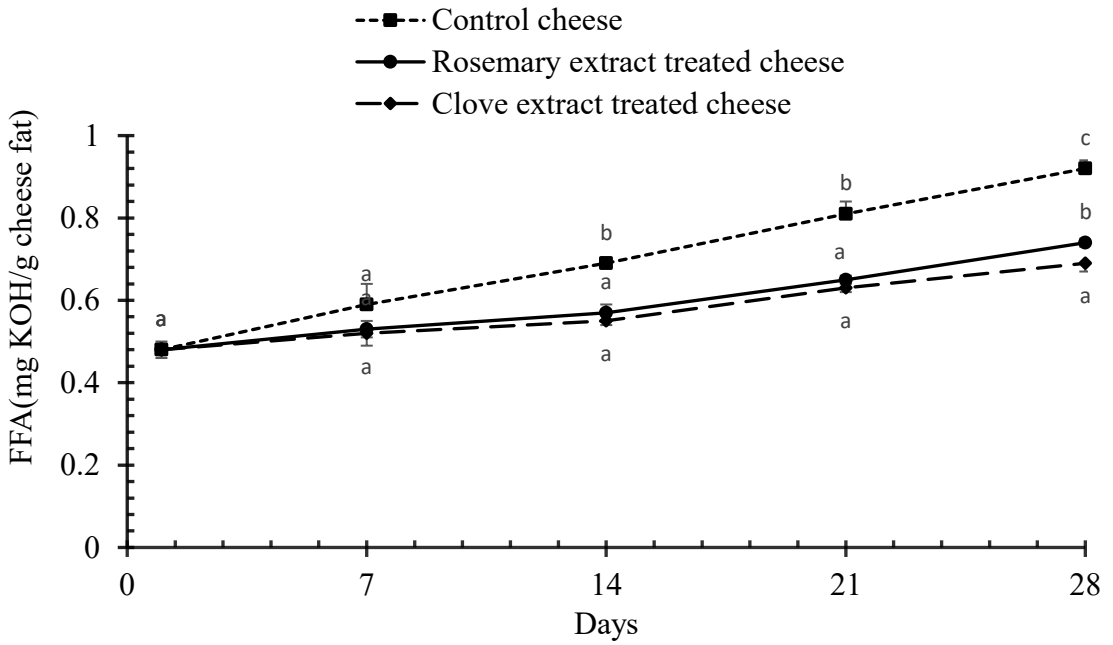


Fig.4.4 Free fatty acid of control and extract incorporated cheeses throughout 28 days of ripening.

Note: Data represents mean ± standard deviation and different superscript are significant different at 5% level of significance

Fig.4.4 shows the level of FFA produced in control and extract treated cheese throughout the 28 days of storage period. The free fatty acid (FFA) values of control and extract incorporated cheeses increased with increasing storage period. The FFA values for control as well as rosemary and clove extract incorporated samples was similar up to 7 days of storage, then the values for control cheese rise at a significantly ($P<0.05$) higher rate than in

extract incorporated samples. The control samples showed the highest values of FFA whereas clove treated cheese had the lowest values in comparison to rosemary and control during 28 days of storage. This might be due to the presence of high eugenol content in clove extract which has the strongest antioxidant and antimicrobial activities as compared to rosemary extract, which might be responsible for lower FFA level compared to rosemary extract. The result obtained in this study was in agreement with El-Aziz *et al.* (2012) who found that soft cheese samples treated with ginger extract had showed lower values of FFAs content than the control cheese.

4.10 Microbiological analysis during storage

Changes in microbiological properties of cheese samples during storage at 4±1ºC were shown in Table 4.4

Table 4.4 Microbiological changes of soft cheeses during storage

Microbial groups (log CFU/g)	Storage days	Treatments		
		Control cheese	Rosemary extract treated cheese	Clove extract treated cheese
Total viable count	1	3.41±0.09^{c}	2.91±0.03^{b}	2.72±0.07^{a}
	7	4.32±0.02^{c}	3.30±0.06^{a}	3.53±0.04^{b}
	14	5.40±0.07^{c}	3.61±0.01^{a}	3.89±0.03^{b}
	21	5.91±0.04^{c}	4.13±0.04^{a}	4.54±0.05^{b}
	28	6.57±0.07^{c}	4.84±0.04^{a}	5.51±0.01^{b}
Yeasts and molds	1	2.54±0.01^{c}	2.15±0.02^{b}	2.10±0.02^{a}
	7	3.24±0.02^{c}	2.59±0.01^{b}	2.38±0.03^{a}
	14	3.87±0.01^{c}	2.98±0.02^{b}	2.76±0.01^{a}
	21	4.99±0.05^{c}	3.63±0.03^{b}	3.42±0.01^{a}
	28	6.11±0.01^{c}	4.79±0.05^{b}	4.14±0.02^{a}

Note. Values are means ± standard deviation of three determinations. Values in rows having different superscript are significant different at 5% level of significance.

The total viable count of all cheese increased significantly ($P<0.05$) during 28 days of storage period. Cheese samples incorporated with rosemary and clove extracts had lower bacterial counts than control sample at refrigerated temperature. This might be due to phenolic compounds and other bioactive components found in spices extracts, which damage microbial cell walls, inhibit enzyme function, and disrupt microbial DNA synthesis (Shan *et al.*, 2011). After 21 days storage, the total viable counts reached 6.57 log CFU/g of the control cheese and 4.84 and 5.51 log CFU/g of the cheese incorporated with rosemary and clove extracts respectively which showed that rosemary had the higher antibacterial activity than the clove extract. The result obtained in the present study are in agreement with Algarni (2016) who reported that the soft cheese supplemented with thyme, cumin and turmeric herbs had the overall lower total bacterial count than the control cheese.

Mold and yeast count of all the cheese samples increased significantly ($P<0.05$) up to the end of storage period. It might be due to lower pH values that may support the growth of yeast and molds (Karam-Allah *et al.*, 2024). Clove extracts incorporated cheese had significantly ($P<0.05$) lower yeast and mold counts than control and rosemary extract incorporated cheese. The general trend of these results are in agreement with those reported by Al-Jasser and Al-Dogan (2009).

All the cheese samples were completely free of the coliform bacteria throughout the storage period. This could be attributed to effective heat treatment and cleanliness practices during cheese manufacturing and storage. This may also confirm the antimicrobial activity of the extract tested in the present study (Saleh, 2018).

4.11 Sensory evaluation of cheeses during storage

The sensory evaluation of cheeses throughout 28 days of storage at 4±1°C is shown in the Table 4.5

Table 4.5 Changes of sensorial properties of cheeses during storage

Parameters	Storage days	Treatments		
		Control cheese	Rosemary extract treated cheese	Clove extract treated cheese
Appearance	1	4.70±0.26[a]	4.63±0.16[a]	4.61±0.23[a]
	7	4.51 ±0.24[a]	4.40±0.18[a]	4.35±0.17[a]
	14	4.45 ±0.15[a]	4.39±0.29[a]	4.23±0.12[a]
	21	4.21±0.29[a]	4.18±0.37[a]	4.11±0.76[a]
	28	3.95±0.56[a]	3.65±0.25[a]	3.55±0.2[a]
Texture	1	4.63±0.36[a]	4.67±0.32[a]	4.65±0.20[a]
	7	4.47±0.25[a]	4.53±0.60[a]	4.50±0.55[a]
	14	4.25±0.25[a]	4.35±0.35[a]	4.32±0.42[a]
	21	3.95±0.40[a]	4.21±0.61[a]	4.15±0.15[a]
	28	3.31±0.36[a]	3.92±0.12[b]	3.85±0.75[b]
Taste	1	4.80±0.20[a]	4.70±0.25[a]	4.60±0.20[a]
	7	4.63±0.23[a]	4.51±0.41[a]	4.48±0.28[a]
	14	4.21±0.10[a]	4.05±0.12[a]	4.00±0.35[a]
	21	4.03±0.43[a]	3.75±0.15[a]	3.55±0.05[a]
	28	3.81±0.36[b]	3.30±0.15[a]	3.19±0.14[a]
Odor	1	4.61±0.29[a]	4.50±0.40[a]	4.43±0.40[a]
	7	4.38±0.37[a]	4.35±0.32[a]	4.28±0.22[a]

	14	4.24±0.31[a]	4.16±0.34[a]	4.12±0.54[a]
	21	3.99±0.05[a]	3.87±0.56[a]	3.80±0.05[a]
	28	3.59 ±0.39[a]	3.41±0.51[a]	3.32±0.7[a]
Aftertaste	1	4.86±0.12[a]	4.75±0.14[a]	4.71±0.29[a]
	7	4.43±0.45[a]	4.11±0.35[a]	4.07±0.32[a]
	14	4.11±0.16[a]	3.98±0.48[a]	3.85±0.65[a]
	21	3.89±0.14[a]	3.57±0.22[a]	3.44±0.06[a]
	28	3.55±0.25[b]	3.13±0.13[ab]	3.05±0.15[a]
Overall acceptability	1	4.73±0.15[a]	4.66±0.09[a]	4.60±0.04[a]
	7	4.48±0.06[a]	4.38±0.20[a]	4.33±0.27[a]
	14	4.25±0.19[a]	4.18±0.23[a]	4.10±0.40[a]
	21	4.01±0.26[a]	3.91±0.38[a]	3.81±0.21[a]
	28	3.64±0.38[a]	3.48±0.23[a]	3.39±0.38[a]

Note. Values are means ± standard deviation of three determinations. Values in rows having different superscript are significant different at 5% level of significance.

The mean score for appearance, texture, taste, and aftertaste decreased with increasing storage period for all samples. Similar result was reported by El-Kholy *et al.* (2017) who studied the effect of essential oils of cumin, rosemary, thyme and their mixture on the quality of UF-soft cheese during storage.

The appearance score of control, extract incorporated cheeses decreased during the storage period and a and no significant difference ($P>0.05$) was observed in control and extract incorporated cheese throughout 28 days of storage period. The results showed that the values decreased as the storage period increased. The findings are in agreement with those of Ahmed and Foda (2012) who observed that as the storage period proceed the appearance of the cheese decreased.

The texture score of control and extract incorporated cheeses decreased during storage

the significant difference ($P<0.05$) after 21 days. The decrease rate was higher for control compared to the other treatments at the end of storage period. This could be due to the higher rate of proteolysis in control cheese which makes the cheese more softer in texture (Al-Jasser and Al-Dogan, 2009).

No significant difference ($P>0.05$) on mean score for taste was observed up to 21 days of storage whereas there was significant difference ($P<0.05$) in cheese samples after 21 days of storage, control cheese being the highly acceptable cheese than extract treated cheeses. Extract treated cheeses introduced the strong flavors by the end of storage period which was less appealing for the sensory panelists. Similar observation was reported by Popescu *et al.* (2023) who demonstrated that taste score decrease significantly ($P<0.05$) by around day 21 in the microencapsulated basil extract incorporated cream cheese.

The mean score for odor of control and extract treated cheese decreased during storage and no significant difference ($P<0.05$) was observed between them throughout storage days. However, control cheese had higher mean odor score than extract incorporated cheese samples that might be due to their strong inherent aromas and breakdown products of extracts during storage which interfere with natural cheese odor over time. Similar observation was reported by (Hamasadek *et al.*, 2023).

Aftertaste is an important sensory parameter in evaluating quality of cheese, as it significantly influences overall acceptability of cheese. The mean score for aftertaste of all cheese samples decreased during storage period with a significant difference ($P<0.05$) after 21 days. The lower mean score was observed for the extracts incorporated samples than the control cheese which may be due to the strong, lingering flavors and potential bitterness of their essential oils, which can be perceived as unpleasant by sensory panelists (Schlossareck and Ross, 2020).

The mean sensory score for overall acceptability of control, rosemary and clove incorporated cheese decreased during storage period of 28 days and no significant difference ($P<0.05$) was observed between control and extract incorporated cheese throughout the storage period. Ibrahim *et al.* (2021) concluded that the incorporation of lower concentration of essential oils or extract into cheese appeared to maintain the overall acceptability as well as sensory properties comparable to control cheese samples manufactured and stored under same condition.

Part V

Conclusions and recommendations

5.1 Conclusions

On the basis of this research, the following conclusions can be made:

1. Extraction yield of rosemary and clove was found to be 18.89% and 36.45% respectively.
2. Total phenolic content, total flavonoid content and antioxidant activity of rosemary extract was found to be 36.11mg GAE/g, 8.02 mg quercetin/g and 68.51% respectively and those of clove extract was found to be 52.88 mg GAE/g, 13.56mg quercetin/g and 75.14% respectively.
3. From the sensory evaluation, soft cheese containing 0.2% rosemary extract and 0.1% clove extract was selected as best cheese.
4. The chemical parameters (fat, protein, and acidity) increased whereas moisture content and pH of control as well as extract treated cheese decreased throughout the storage period of 28 days.
5. The free amino acid of all cheese samples increased with increase in storage period with significantly ($P<0.05$) lower value for clove incorporated cheese after 7 days.
6. The total free fatty acid of all cheese samples increased with increase in storage with significantly ($P<0.05$) lower value for clove extract incorporated cheese during 28 days of storage.
7. The total viable counts were significantly($P<0.05$) lower in rosemary extract incorporated cheese than control and clove extract incorporated cheese whereas yeasts and molds counts of clove extract incorporated cheese were significantly ($P<0.05$) lower than control and rosemary extract incorporated cheese.
8. Throughout the 28 days of storage, a significant difference ($P<0.05$) was observed between control and extracts incorporated samples in terms of texture, taste and aftertaste. However, no significant differences ($P>0.05$) were found in other sensory attributes, including appearance, odor and overall acceptability between control and extracts incorporated samples.

5.2 Recommendations

Based on the present study, the following recommendations can be made:

1. Other herbs and spices such as mints, lemongrass, cinnamon could be examined for their potential to act as preservatives in soft cheese.
2. Other extraction methods such as. ultrasound, microwave, enzyme-assisted, or supercritical fluid extraction could be explored to enhance yield and efficiency

Part VI

Summary

Cheese is a nutrient-rich dairy product which is very prone to microbial spoilage and subsequent biochemical deterioration. Herbs and spices are added in cheese to increase the stability of cheese. The primary objective of this research work is to study the effect of incorporating rosemary and clove extracts on the quality and shelf-life characteristics of soft cheese. The ethanolic extract of rosemary and clove was prepared separately and they are analyzed for total phenolic content, total flavonoid content and antioxidant activity. Soft cheeses were prepared by incorporating five different concentrations (0.1%, 0.2%, 0.3%, 0.4% and 0.5%) of rosemary and clove extract individually while control cheese (0% extracts) was also prepared. The best formulation was selected based on sensory evaluation. The selected and control cheese were analyzed for physicochemical, microbiological, and sensory properties over 28 days at 7-day intervals.

The total phenolic content, total flavonoid content and antioxidant activity of rosemary extract was found to be 36.11 mg GAE/g, 8.02 mg quercetin/g and 68.51% and those of clove extracts was 52.88 mg GAE/g, 13.56 mg quercetin/g and 75.14%. Soft cheese incorporated with 0.2% of rosemary extract and 0.1% clove extract was found to be significantly ($P<0.05$) superior in terms of sensory quality. Addition of rosemary and cloves extract and storage days significantly affected the chemical characteristics of soft cheese samples during storage. Throughout the storage period, control cheese exhibited lower moisture and pH content but higher fat, ash and acidity compared to extract incorporated cheese. However, there was no significant difference ($P>0.05$) among protein content of each samples including control cheese. Control cheese had significantly ($P<0.05$) higher free amino acid after 7 days and rosemary extract incorporated cheese after 21 days than that treated with cloves extract. Extracts incorporated cheese had significantly ($P<0.05$) lower free fatty acid content than control cheese. The total viable counts were significantly ($P<0.05$) higher in control cheese and lower in rosemary extract incorporated cheese. The yeasts and molds counts were significantly ($P<0.05$) lower in clove extract incorporated cheese and higher in control cheese. Coliforms were not detected in either sample. During 28 days of storage, a significant difference ($P<0.05$) was observed between control and extracts incorporated samples in terms of texture, taste and aftertaste. However, no

significant differences (P>0.05) were found in other sensory attributes, including appearance, odor and overall acceptability.

It was concluded that the incorporation of 0.2% rosemary extract and 0.1% clove extract preserved the physicochemical and microbiological quality of soft cheese with the minimal adverse sensory effects within 28 days of storage period.

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Appendices

Appendix A

Specimen card for sensory evaluation

Hedonic rating test

Date: ……...

Name of panelist: ……...

Product: Soft cheese

Please conduct the sensory analysis based on the following parameter using the scale given. Panelists are requested to give ranks on their individual choice.

Excellent 5

Good 4

Satisfactory 3

Fair 2

Poor 1

Samples	**Parameters**					
	Appearance	Texture	Flavor	Odor	Aftertaste	Overall Acceptability
A						
B						
C						

Comment (if any): …….

…….

Signature: …….

Appendix B

ANOVA table for mean comparisons on free amino acid of cheese samples treated with extracts of rosemary and clove and control cheese during storage

Table B.1 ANOVA table for mean comparisons on free amino acid of cheese samples treated with extracts of rosemary and clove and control cheese during storage

Day 1	Sum of squares	df	Mean square	F	Sig.
Between Groups	0.000	2	0.000	0.500	0.630
Within Groups	0.001	6	0.000		
Total	0.001	8			
Day 7					
Between Groups	0.027	2	0.014	33.108	<0.001
Within Groups	0.002	6	0.000		
Total	0.30	8			
Day 14					
Between Groups	1.429	2	0.715	739.345	<0.001
Within Groups	0.006	6	0.001		
Total	1.435	8			
Day 21					
Between Groups	2.795	2	1.398	6987.500	<0.001
Within Groups	0.001	6	0.000		
Total	2.796	8			
Day 28					
Between groups	3.745	2	1.872	9362.000	<0.001
Within groups	0.001	6	0.000		
Total	3.746	8			

Appendix C

Tukey HSD analysis of free amino acid of cheese treated with clove and rosemary extract during storage

Table C.1 HSD test for Day 1

Treatment	N	Subset alpha=0.05
		a
Rosemary	3	1.4200
Clove	3	1.4200
Control	3	1.4300
Sig.		0.679

Mean for groups in homogenous subsets are displayed.

a. Uses Harmonic Mean Sample Size=3.000

Table C.2 HSD test for Day 7

Treatments	N	Subset for alpha=0.05	
		a	b
Clove	3	1.5900	
Rosemary	3	1.6400	
Control	3		1.7233
Sig.		0.053	1.000

Mean for groups in homogenous subsets are displayed.

a. Uses Harmonic Mean Sample Size=3.000

Table C.3 HSD test for Day 14

Treatments	N	Subset for alpha=0.05	
		a	b
Clove	3	1.8900	
Rosemary	3	1.9200	
Control	3		2.7500
Sig.		0.505	1.000

Mean for groups in homogenous subsets are displayed.

a. Uses Harmonic Mean Sample Size=3.000

Table C.4 HSD test for Day 21

Treatments	N	Subset for alpha=0.05		
		a	b	c
Clove	3	2.6900		
Rosemary	3		2.8400	
Control	3			3.9400
Sig.		1.000	1.000	1.000

Mean for groups in homogenous subsets are displayed.

a. Uses Harmonic Mean Sample Size=3.000

Table C.5 HSD test for Day 28

Treatments	N	Subset for alpha=0.05		
		a	b	c
Clove	3	4.3500		
Rosemary	3		4.6700	
Control	3			5.8500
Sig.		1.000	1.000	1.000

Mean for groups in homogenous subsets are displayed.

a. Uses Harmonic Mean Sample Size=3.000

Appendix D

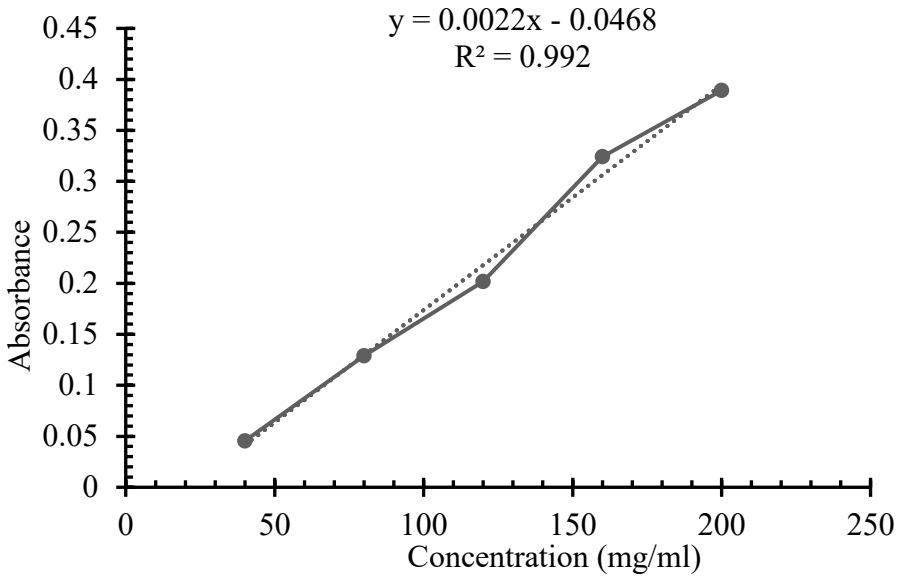

Fig. D.1 Standard curve for total phenolic content

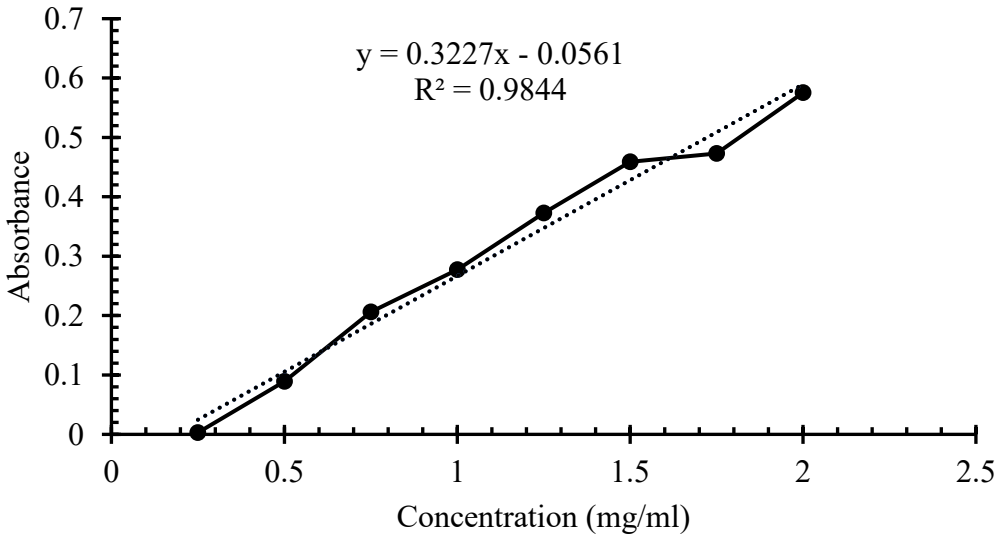

Fig. D.2 Standard curve for flavonoid content

Appendix E

List of equipment used:

Hot air oven

Grinder

Refrigerator

Heating arrangement

Heating vessels

Gerber butyrometer

Titration set

Muffle furnace

Weighing balance

Bacterial Incubator

pH meter

Beakers

Thermometer

Volumetric flasks

Pipettes and micropipettes

Petri plates

Water bath

Magnetic stirrer

Spectrophotometer

Rotary vacuum evaporator

Colony counter

Autoclave

Kjeldahl digestion and distillation set

List of Chemicals:

Hydrochloric acid

Phenolphthalein

Isoamyl alcohol

Potassium sulphate

Methanol

DPPH (Diphenyl-picrylhydrazine)

Ferric chloride

Gallic acid

Ethanol

F-C reagent

Quercetin

Sodium nitrate

Aluminum chloride

Sodium Carbonate

Diethyl ether

Potassium hydroxide

Sodium sulfate

Phenolphthalein

Potato dextrose agar

Plate count agar

Red blue agar

Ninhydrin

Glacial acetic acid

Cadmium chloride

Color plates

Plate P.1 Rosemary and clove extract

Plate P.2 Cutting cheese coagulum

Plate P.3 Sensory analysis of cheese

Plate P.4 Microbial analysis

Plate P.5 Cheese samples for analysis

Plate P.6 Protein determination by Kjeldahl