SVEUČILIŠTE JOSIPA JURJA STROSSMAYERA U OSIJEKU

PREHRAMBENO-TEHNOLOŠKI FAKULTET OSIJEK

Almir Abdurramani

UTJECAJ NADTLAKA NA MIKROBIOLOŠKA SVOJSTVA I AROMATIČNE KOMPONENTE POLUTVRDOG SIRA TIJEKOM ZRENJA U POSEBNO DIZAJNIRANIM KOMORAMA ZA ZRENJE

DOKTORSKI RAD

Osijek, 2026.

JOSIP JURAJ STROSSMAYER UNIVERSITY OF OSIJEK

FACULTY OF FOOD TECHNOLOGY OSIJEK

Almir Abdurramani

INFLUENCE OF OVERPRESSURE ON MICROBIOLOGICAL PROPERTIES AND AROMA COMPONENTS OF SEMI-HARD CHEESE DURING RIPENING IN SPECIALLY DESIGNED RIPENING CHAMBERS

DOCTORAL THESIS

Osijek, 2026.

TEMELJNA DOKUMENTACIJSKA KARTICA
DOKTORSKI RAD
Sveučilište Josipa Jurja Strossmayera u Osijeku
Prehrambeno-tehnološki fakultet Osijek
Doktorski studij Prehrambena tehnologija i nutricionizam
Smjer: Prehrambena tehnologija

Zavod za prehrambene tehnologije
Katedra za tehnologiju ugljikohidrata
Franje Kuhača 18, 31000 Osijek, Hrvatska
UDK:
Znanstveno područje: **Biotehničke znanosti**
Znanstveno polje: **Prehrambena tehnologija**
Znanstvena grana **Inženjerstvo**
Tema rada **prihvaćena je na X. sjednici Fakultetskog vijeća Prehrambeno-tehnološkog fakulteta Osijek održanoj 24. 7. 2024.**
Mentor: **Izv. prof. dr. sc. Marijana Blažić**
Komentor: **Prof. dr. sc. Jurislav Babić**

UTJECAJ NADTLAKA NA MIKROBIOLOŠKA SVOJSTVA I AROMATIČNE KOMPONENTE POLUTVRDOG SIRA TIJEKOM ZRENJA U POSEBNO DIZAJNIRANIM KOMORAMA ZA ZRENJE
Almir Abdurramani, 0113145878/2019.

Sažetak:
Cilj ovog istraživanja bio je utvrditi kako specifični uvjeti zrenja, osobito nadtlak, utječu na sazrijevanje sira i kvalitetu konačnog proizvoda. Posebno konstruirane komore za zrenje omogućile su preciznu regulaciju temperature, relativne vlažnosti, strujanja zraka i nadtlaka. Uzorci sira analizirani su mikrobiološki, GC/MS metodom, fizikalno-kemijski i senzorski tijekom dva mjeseca (10–16 °C, 75–85 % relativne vlažnosti). Utjecaj trajanja zrenja (3,59–6,41 tjedana) i nadtlaka (1,18–3,31 mbar) ispitan je metodologijom odzivne površine (RSM). Rezultati su pokazali da povišeni tlak učinkovito regulira rast bakterija i kvasaca, dok je količina plijesni pokazivala dvofazni trend – početni porast praćen smanjenjem prema kraju zrenja. GC/MS analizom utvrđeno je da je 3,5-dihidroksidekanska kiselina 1,5-lakton najzastupljenija aromatična komponenta. Fizikalno-kemijski parametri ostali su unutar propisanih standarda, a senzorska procjena potvrdila je više ocjene arome i prihvatljivosti sireva zrelih pod nadtlakom. Zaključeno je da primjena komora za zrenje s nadtlakom može značajno unaprijediti mikrobiološku stabilnost i aromatski profil sira, čime se poboljšava njegova ukupna kvaliteta.

Ključne riječi: sir, zrenje, nadtlak, aroma, optimizacija

Rad sadrži:
96 stranica
17 slika
11 tablica
0 priloga
69 literaturnih referenci

Jezik izvornika: **Engleski**

Sastav Povjerenstva za ocjenu:

1.	**izv. prof. dr. sc. Mirela Lučan Čolić**	**predsjednik**
2.	**izv. prof. dr. sc. Krunoslav Aladić**	**član**
3.	**izv. prof. dr. sc. Irena Barukčić Jurina**	**član**
4.	**dr. sc. Irena Perković, dipl.ing.**	**zamjena člana**

Datum obrane:

BASIC DOCUMENTATION CARD
DOCTORAL THESIS

Josip Juraj Strossmayer University of Osijek
Faculty of Food Technology Osijek
Doctoral Study Food Technology and Nutrition

Department of Food Technology
Subdepartment of Carbohydrates Technology
Franje Kuhača 18, HR-31000 Osijek, Croatia

UDC:
Scientific area: **Biotechnical sciences**
Scientific field: **Food technology**

Thesis subject **was approved by the Faculty Council of the Faculty of Food Technology at its session no. X held on 24th July 2024.**
Mentor: **Marijana Blažić, PhD, assistant prof.**
Co-mentor: **Jurislav Babić, full prof.**

INFLUENCE OF OVERPRESSURE ON MICROBIOLOGICAL PROPERTIES AND AROMA COMPONENTS OF SEMI-HARD CHEESE DURING RIPENING IN SPECIALLY DESIGNED RIPENING CHAMBERS

Almir Abdurramani, 0113145878

Summary:
The aim of this study was to investigate the influence of ripening conditions, particularly overpressure, on cheese maturation and final product quality. Specially engineered ripening chambers enabled precise control of temperature, relative humidity, air circulation, and overpressure. Cheese samples were subjected to microbiological testing, GC/MS analysis of volatile compounds, physicochemical analysis, and sensory evaluation over a two-month period (10–16 °C, 75–85% relative humidity). The impact of ripening duration (3.59–6.41 weeks) and overpressure levels (1.18–3.31 mbar) was evaluated using response surface methodology (RSM). Results showed that increased pressure effectively limited bacterial and yeast growth, while mould levels initially rose before declining toward the end of maturation. GC/MS analysis identified 3,5-dihydroxydecanoic acid 1,5-lactone as the predominant aromatic compound. Physicochemical parameters remained within prescribed standards, and sensory evaluation confirmed superior flavour and aroma in overpressure-ripened cheeses. Overall, the findings suggest that overpressure in specialized ripening chambers can markedly enhance microbial stability and improve the aromatic profile of cheese, contributing to better overall product quality.

Key words: **cheese, ripening, overpressure, aroma, optimization**

Thesis contains: **96 pages**
17 figures
11 tables
0 supplements
69 references

Original in: **English**

Review (Evaluation) committee:

1.	**Mirela Lučan Čolić, PhD, assistant prof.**	**chair person**
2.	**Krunoslav Aladić, PhD, assistant prof.**	**member**
3.	**Irena Barukčić Jurina, PhD, assistant prof.**	**member**
4.	**Irena Perković, PhD**	**stand-in**

Defense date: **date**

Printed and electronic (pdf format) version of thesis is deposited in Library of the Faculty of Food Technology Osijek, University of Osijek; Rector's office, University of Osijek; University Library in Zagreb and in University Library in Osijek

DOKTORSKI RAD JAVNO JE OBRANJEN DANA

_____

Pred Povjerenstvom za obranu doktorskog rada:

1. **izv. prof. dr. sc. Mirela Lučan Čolić** predsjednik _____ (potpis)

2. **izv. prof. dr. sc. Krunoslav Aladić** član _____ (potpis)

3. **izv. prof. dr. sc. Irena Barukčić Jurina** član _____ (potpis)

CONTENTS

1. INTRODUCTION

Cheese ripening is widely recognised as the longest, most complex and least controllable stage of cheese production, governed by intertwined biochemical processes (glycolysis, proteolysis and lipolysis) that ultimately determine the flavour, aroma, texture and safety of the final product. Because the outcome of ripening is difficult to predict and standardise, producers continuously seek tools capable of shortening the ripening period, improving product consistency, and enhancing microbiological safety without resorting to additives or thermal treatments that compromise the natural quality of cheese (Zheng *et al*., 2021). Various ripening aids, such as protective foils and edible coatings, exogenous enzymes, attenuated starter cultures, ozonisation, and modified-atmosphere packaging, have already been investigated with this aim in mind, each offering certain advantages while also bearing specific technological or sensory limitations (Guinee and O'Brien, 2010).

High-pressure processing (HPP) has been established as an effective non-thermal method for inactivating undesirable microorganisms and modulating enzymatic activity in milk and cheese and has already been applied at several stages of cheese manufacture, namely for milk pasteurisation, during curd formation, shortly after manufacture, and following the ripening stage (Gervilla *et al.*, 2000). However, despite this substantial body of evidence, the application of continuous mild overpressure within a specially designed pressurised ripening chamber, maintained throughout the entire maturation process itself, has not yet been investigated. This gap between the well-documented potential of high pressure and its unexplored use as a continuous ripening-environment parameter constitutes the central research problem addressed in this work, and is justified both scientifically, by the absence of comparable data in the literature, and economically, by the potential of such technology to shorten ripening time and improve product quality and safety for cheese producers Calzada *et al.* (2015a).

Accordingly, the aim of this research was twofold: (a) to determine the optimal conditions for ripening semi-hard cheese in an overpressure chamber, based on the combination of overpressure and ripening time yielding the most favourable maturation profile, as verified by GC–MS analysis of volatile aroma compounds; and (b) to evaluate the microbiological safety of the production and ripening process through continuous monitoring of yeasts, moulds, *Listeria monocytogenes* and *Salmonella* spp. To achieve these aims, semi-hard cheese was manufactured from pasteurised cow's milk and ripened in parallel in two specially designed chambers: a control chamber operating at atmospheric pressure and an overpressure chamber allowing controlled overpressure in the range of 0.1 – 5.0 mbar. The combined effect of overpressure (1.18 – 3.31 mbar) and ripening time (3.59 – 6.41 weeks) was optimised using

response surface methodology (RSM) based on a central composite rotatable design (CCRD). Volatile aroma compounds were identified and quantified by gas chromatography–mass spectrometry (GC-MS) coupled with solid-phase microextraction (SPME); microbiological quality was assessed in accordance with the relevant ISO/HRN standards; physicochemical composition (fat, moisture, protein and salt content) was determined by near-infrared spectroscopy; and sensory quality was evaluated by a trained panel using quantitative descriptive analysis (QDA).

On the basis of the foregoing, the basic hypothesis of this research was that the application of continuous mild overpressure during the ripening of semi-hard cheese, under controlled conditions in a specially designed overpressure ripening chamber, significantly influences the formation of volatile aroma compounds and the microbiological, sensory and physicochemical quality of the cheese in comparison with cheese ripened under atmospheric pressure, and that, by means of response surface methodology, an optimal combination of overpressure and ripening time can be identified that simultaneously improves cheese quality and microbiological safety without the use of additives or extensive manual intervention.

2. THEORETICAL PART

Based on its characteristics, cheese is a wholesome foodstuff that provides all dietary, nutritional and functional benefits and, in addition, it also satisfies the gourmet aspect of food consumption. As a result of consumer demands for healthier and more functional foods, the global value of the cheese market in 2022 was more than 200 billion dollars. The beginning of cheese production and consumption date back several thousand years and its popularity has not waned, as evidenced by the fact that cheese is still an indispensable food item in every household. Since its early stages, the technology of cheese making has evolved due to the continuous development of new materials, equipment and production methods. Currently, modern cheese production is at its high level concerning quality and incorporated technology. But in terms of cheese quality, it is highly influenced by the consumers liking and dependable on the properties and quality of milk supply, and the production technology (Oštarić *et al*., 2022). The properties of the raw milk have the most significant influence on the quality of the cheese. The quality of cheese is influenced by various factors such as the composition and quality of milk, including microbial quality, enzymatic activity, somatic cell count, and levels of residues or contaminants. The concentrations of protein, casein, and fat in cheese milk significantly impact several aspects of cheese making like rennet coagulability, gel strength, curd syneresis, cheese yield, and overall quality. Raw milk properties and composition depend on animal genotypic and phenotypic characteristics, nutrition and feed, stage of lactation, seasons, they even depend on the frequency of milking (Guinee and O'Brien, 2010). Raw milk must be of good microbial quality, which is due to appropriate hygiene on the farm and during the handling of milk. It must have a low total count of bacteria, be absent from pathogenic and harmful bacteria and, as low as possible, count of psychotropic bacteria, which produce heat-resistant enzymes (proteases and lipases) that can reduce yield and cause off-flavours in mature cheese (Skeie, 2010). A high number of somatic cells can be an indicator of mastitis in an animal and can cause a reduction of lactose, fat and casein in milk which affect gel strength, protein recovery from milk to cheese and, ultimately, cheese yield. Proteinase and lipase, such as plasmin, lysozyme, cathepsin D, lipoprotein lipase, etc., present in milk can originate from a number of sources (milk, bacteria, somatic cells) who can have a significant effect on cheesemaking properties, yield and quality. The presence of contaminants and chemical residues in milk can have both economical and technological impact on the dairy industry. Veterinary residues (antibiotics), cleaning and disinfecting agents and compounded animal feeds (mycotoxins) can cause inhibition of non-starter or/and added starter cultures in milk which can lead to inadequate ripening and ageing of cheese and, consequently, defects of flavour and texture (Guinee and O'Brien, 2010). As previously stated, the quality of milk must

be high, which also includes its sensory characteristics. Milk should not have offbeat appearance, colour and unpleasant smells and tastes that will directly affect the quality of the final product. Other than that, cheese quality and yield is influenced by the milk handling, cold storage, pre-treatments that include milk standardization and heat treatments. Since cheese is the end product of a very complex and long process, it is important to anticipate possible problems and their effective solutions during its manufacture.

Generally, we can establish desirable cheese parameters that we want to achieve (e.g. flavour or texture) and according to that modify manufacturing and ripening protocols. However, throughout cheese production, there are processes that are not fully controllable. Although the process of making cheese is relatively straightforward, it encompasses intricate chemical and physical events (Guinee and O'Brien, 2010). First step in cheese production, assuming that the milk has been pre-treated, is milk coagulation. Milk can be coagulated by lowering the pH value (acidification), which can occur spontaneously by indigenous non-starter bacteria, intentionally by the addition of starter cultures or organic acids. PH at 4.6 causes precipitation of the casein micelles to aggregate and form a clot. This way of curdling is typical to fresh cheeses and some traditional cheeses, since it was one of the first methods of cheesemaking. Furthermore, deliberately added starter culture is also used to enhance the aroma, taste and texture of the cheese and to inhibit the growth of unwanted bacteria. Using coagulating enzymes is another way of coagulating milk and is applied for the production of most cheese varieties. The oldest technological application of enzymes dates back to around 5000 BC, which proves the intentional conversion of milk into cheese in order to extend its shelf life, making it safer for consumption (Oštarić *et al*., 2022). Nowadays, there are a lot of options to choose from, coagulants obtained from stomach of young ruminants (rennet) or using plant, fungal or microbiological coagulants, they all serve the same purpose - destabilization of the casein colloidal suspension of micelles and their aggregation forming a stable gel network. The optimal temperature range for the formation of curd is between 30 - 35 °C, which provides favourable conditions for the activity of the starter culture and enzymes. A network of proteins, fat, whey and minerals is formed. By cutting the curd and reheating the cheese grains we are controlling cheese firmness, that is, retention of whey, recovery of fat and proteins. The size of the curd after cutting is determined by the type of cheese. Cheese grain that needs to be reheated at higher temperatures is cut into smaller pieces, while grain that is reheated at lower temperatures is cut into larger pieces. The smaller the size of the curd particles, the more whey and fat is lost, and vice versa. At this point, an increase in temperature and acidity of the

environment leads to syneresis. Syneresis is the process of rearranging casein molecules, which results in tightening of the protein network causing whey separation. As already mentioned, with whey syneresis the water phase is being extracted, and with it lactose, whey protein and dissolved salt leading to the concentration of fat and protein. There are technological procedures that conditions the release of more whey, depending on the type of cheese. At this point, it is possible to add salt, to further stimulate syneresis, or the salt can be added after the whey drainage directly in curd, by cheese wheels soaking in brine or by rubbing salt at cheese surface during ripening. When dry salt is rubbed on cheese surface, a rind is formed that over time becomes greasy due to fat migration. Generally, rind prevents moisture loss so that the cheese does not become dry. Moisture cannot easily move through the dense casein network, but neither can salt. Therefore, dry-salted cheeses can also be brined or salted before pressing. By pressing the curd, cheese is shaped into the desired shape, whey drainage is accelerated, and finally, under pressure, the curd forms faster and better (Guinee and O'Brien, 2010). All of the mentioned processes have an impact on the cheese ripening. The non-starter bacteria (indigenous or got into milk or cheese during its production) and starter cultures of bacteria continue their activity during maturing of cheese, rennet used to coagulate milk is the principal proteolytic agent during ripening, the procedures of cutting the curd, reheating the cheese grain, separating the whey, pressing and salting affect the amount of water in cheese, and depending on the moisture content, cheese preservation and ripening methods can vary greatly (Zheng *et al*., 2021).

Cheese quality can be described as a set of properties that give value to cheese, these include appearance, taste, smell, texture, nutritional value, functionality and safety. During cheese production, many difficulties are encountered that could affect the final quality of the cheese. Firstly, if the milk doesn't coagulate, the reason for that could be the absence of indigenous bacteria in milk (inadequate thermal treatment), coagulating enzymes (old or unviable rennet) or insufficient quantity of protein and soluble calcium in milk that leads to non-existent or weak curd. In contrast to that, over-acidification (excessive growth of microorganisms in milk) and less amount of proteins can cause cheese to become dry and brittle. The presence of contaminants can also affect curd formation. During the process of cleaning and disinfection of cheese equipment, residues of chemical agents are often left behind, which can end up in the milk during its processing and prevent milk from coagulating. Furthermore, a presence of well-known bacterial virus, bacteriophage, in milk or in starter culture, is responsible for the lysis of bacterial cells, and thus preventing the formation of acid and the

adequate taste and smell of cheese. Improper curd formation can lead to cheese that is too dry, too wet, or lacks the desired texture. Factors such as pH, temperature, and coagulation time can all affect curd formation. However, the most difficulties in cheese production occur during its maturation phase that is why it is important to harmonize parameters such as temperature, humidity, air flow with adequate cheese care. For example, higher temperatures cause rapid aging that can lead to spoilage and off-taste and smell. On the other hand, lower temperatures can retard texture changes not allowing the cheese to properly set. Cheese can be contaminated by bacteria, yeasts, or moulds that can cause spoilage or affect the flavour, aroma, and texture of the cheese or cause uneven ripening. Contamination can occur at any point in the production process, from milk collection to aging and storage. Uncontrolled biochemical processes can result in cheese that is overly soft, slimy, or bitter as a result of proteolysis, or a cheese that is overly oily or rancid by cause of lipolysis. Proteolysis and lipolysis can occur due to the activity of unwanted microorganisms or due to improper aging or storage conditions. To prevent these problems, cheese makers must carefully control the production process, monitor the quality of the milk and cheese, and implement good manufacturing practices to ensure the safety and quality of the final product.

Alternative ways have been proposed to give advantages compared to current manufacturing process and its difficulties. One of them is membrane filtration processes used to standardize ratio of proteins (casein, serum protein), fat and lactose content in milk for cheese production. It is a separation technique where molecules of different size and characteristics are set apart obtaining retentate (milk fat and proteins) and permeate (water, sugar, some minerals and non-protein nitrogen compounds). When using highly concentrated retentate in cheese manufacture, the occurrence of syneresis is reduced, consequently, the textural properties and yield of the cheese are increased due to the higher protein content. Microencapsulation technology has been extensively used in food production. Primary objectives of encapsulation are to protect its contents from external influences (e.g. oxygen, light, moisture, radiation, pH value), preserve them during food processing, distribution and storage, and to ensure the stability while allowing encapsulated active to migrate in and out of the membrane enabling controlled release of the active substance (Timilsena *et al.*, 2020). In terms of cheese processing, economic aspects are in relation to reduced enzyme and ripening time, and improvement of flavour (Mohammadi *et al.*, 2014). Direct addition of enzymes into milk during cheese production leads to their loss with the separation of whey due to their water-soluble property. This problem affects the quality of cheese, but it can be resolved with the encapsulation of

enzymes (Deepali & Waghmare, 2020). By incorporating protective enzymes, such as nisin – a bacteriocin that inhibits pathogens and spoilage microorganisms in cheese without affecting the course of cheesemaking, it is possible to increase the safety of cheese. In addition to nisin being a preservative, it also induces lysis of some bacterial cells. With regards to cheesemaking, it causes a release of starter cultures intracellular enzymes thus accelerating proteolysis and lipolysis which is associated with flavour development. In addition to accelerating cheese ripening, encapsulation technology is mainly used to fortify cheese with bioactive compounds or to increase the shelf-life of cheese products (Oštarić *et al*., 2022).

Beyond ensuring good sensory quality, it is important to keep in mind that cheese can be a potential source of foodborne illness if proper food safety measures are not followed. The foundation of safe cheese production lies in Good Manufacturing Practices (GMPs) and the Hazard Analysis and Critical Control Points (HACCP) system. GMPs encompass a range of procedures and practices designed to ensure that cheese is produced in a safe and hygienic manner, including proper handwashing, cleaning and sanitizing of equipment and surfaces, and temperature control throughout the production process. The HACCP system complements these measures by identifying potential hazards at each stage of production and establishing controls to prevent them, supported by systematic monitoring, corrective actions, and thorough record-keeping.

In addition to these systems, cheese manufacturers routinely conduct testing for pathogenic microorganisms such as Listeria monocytogenes, Salmonella, and E. coli to verify the microbiological safety of their products. Testing is carried out both on the raw milk used in production and on the finished cheese. Proper storage and handling are equally critical. Cheese should be kept at 4°C or below to inhibit bacterial growth, and soft cheeses should be consumed within a few days of opening. Handling should always be carried out with clean hands and utensils to prevent cross-contamination. Finally, accurate labelling plays an essential role in consumer safety. Cheese packaging should clearly indicate the product name, date of production, and expiration date, enabling consumers to identify safe products and avoid consuming cheese that is past its shelf life.

2.1. Cheese Ripening

The consumers acceptability of cheese depends largely on a specific sensory characteristic, of which the most important are taste and aroma. Cheese ripening is a very complex and long process that involves the enzymatic breakdown of the larger curd components

– proteins, carbohydrates, and lipids, into a simpler compounds and molecules that participate in the quality of the cheese. The presence of curd's break-down products, such as, free amino acids, fatty acids, amines, ketones, lactones, ethanol, etc., is directly related to the cheese making process and the conditions during ripening (Zheng *et al*., 2021). The specific properties of almost all types of cheese are determined primarily by chemical and biochemical processes that occur during cheese maturity. Considering chemical parameters; fat, salt, and water content in cheese determine the course of the ripening and the organoleptic properties of the cheese (Kalit, 2015). Biochemical changes of the cheese can be divided into two stages; primary and secondary. Primary biochemical changes include glycolysis, proteolysis, and lipolysis. At the beginning of ripening, different types of cheese have similar mild or neutral organoleptic properties, while the characteristic flavour and aroma of individual cheeses are formed during maturing.

Glycolysis is a shared term for metabolism of residual lactose and metabolism of lactate and citrate. Key factors in metabolism of residual lactose are starter culture of lactic acid bacteria (LAB) who transform lactose into lactic acid, that is, lactate. Predominance of lactose is lost with whey during cheese production, however, low levels of residual lactose still remain in the curd. It is important that starter LAB completes majority of lactose fermentation to prevent the development of secondary microflora that could cause undesirable changes during ripening. The rate of lactose fermentation is largely dependable by ripening temperature and salt content of curd since they affect the activity of starter bacteria (McSweeney, 2004). Generally, ripening process is carried out at low temperatures, between 4 and 18°C and a salt content varying from 1.5 to 2.3%, however, salt is still concentrated in the cheese rind at the start of the ripening period allowing unhindered microbial activity (Tidona *et al*., 2022). By accumulating lactic acid, i.e., fermenting lactose, the pH of the curd lowers to 5.0 – 5.3 within the first 12 hours from the start of cheesemaking process. The cheese quality is highly influenced by the fermentation of residual lactose. At low concentrations of salt (<5%) in cheese and inferior populations of non-starter culture (NSLAB), lactose is converted to L-lactate by the starter culture of LAB. Opposed to that, NSLAB is less sensitive to higher salt concentrations (>5%), and being dominant in cheese, converts lactose to D-lactate, or racemize L-lactate to D-lactate. Considering flavour, DL-lactate is no significantly different, but in the form of calcium D,L-lactate causes white crystalized clusters that which could have unfavourable effect on the sensory acceptability of the product. After all the available carbohydrates have been metabolized, present microbial varieties oxidize lactate. Generally,

starter LAB metabolize lactate into aroma components acetate, ethanol, formate and carbon dioxide, but depending on the starter culture and the type of cheese we want to obtain, the oxidation products are different. Microbial possibility of oxidation is dependable on the availability of oxygen and the size of the cheese. In the absence of oxygen, during anaerobic fermentation, late gas blowing of the cheese can occur, during which lactate is converted into butyrate, H_2 and carbon dioxide by the activity of NSLAB, specifically, *Clostridium tyrobutyricum*. Other than texture deficits, late gas blowing causes undesirable flavours which causes cheese to, ultimately, cannot be for consumption. In order to prevent that, the correct hygiene and heat treatment of the milk must be carried out, measures to prevent subsequent contamination and a sufficient amount of salt in the cheese must be ensured. Low levels of citrate can be found in cheese due to its outflow with whey, but nevertheless, they are important precursors for flavour development. Citrate is metabolized to diacetyl (butane-2,3-dione), acetoin (3-hydroxy-butan-2-one), butane-2,3-diol and carbon dioxide by citrate (+) bacterial strains, *e.g. Lactococcus lactis* ssp. *lactis biovar diacetylactis*, *Leuconostoc mesenteroides* ssp. *cremoris*, and/or *Leuconostoc lactis*, usually present in the starter culture, or some bacterial strains present in NSLAB flora. Other carbohydrates in cheese can also be broken down by microorganism produced enzymes, resulting in the formation of various compounds such as carbon dioxide and acetic acid.

It is known that milk fat is important for the development of many volatile flavours during cheese ripening. The process of breaking down lipids is called lipolysis. Cheese has a low oxidation/reduction potential which causes limited oxidative changes. However, enzymatic hydrolysis of triglycerides produces a wide range of free fatty acids. In cheese curd at the beginning of the ripening stage, indigenous, endogenous and exogenous lipases are present. Naturally occurring lipoprotein lipases are characteristic agents in the production of raw milk cheese due to their sensibility to high heat. In untreated milk, they don't cause changes of milk fat globules because they are surrounded and protected with membrane. However, by applying mechanical methods of milk processing during the production of certain types of cheese, such as homogenization in Blue cheese production, lipids are exposed and subjected to hydrolysis. Homogenisation is not only used to increase the rate of lipolysis, but also to increase cheese yield due to reduced fat loss. The most extensive breakdown of lipids occurs when using natural rennet paste. Stomachs of ruminant cubs are macerated and made into a paste that contains milk-clotting and proteolytic enzymes, chymosin and pepsins in different ratios, and a complex of lipolytic enzymes, among which a very potent lipase, pregastric esterase. Other than that,

microbial lipolytic activity is also present during ripening. Bacterial cells release enzymes that break down triglycerides into long-chain free fatty acids. The most intense bacterial lipolytic activity is recorded in mould- and smear-ripened cheese varieties. *Penicillium* spp. is associated with extensive lipolytic activity that does not require cell lysis, as is the case for LAB and NSLAB strains. However, starter and non-starter cultures during semi-hard cheese ripening are present in a large number for a long period of time, and with the release of lipolytic enzymes, can cause a significant level of lipolysis (McSweeney, 2004). Long-chain free fatty acids do not contribute significantly to the aroma and flavour of the cheese. Released medium- and short-chain free fatty acids, as they are, directly contribute to the cheese flavour, but they also act as precursors for the development of other taste and flavour components.

Bacterial autolysis occurs during the unfavourable conditions, such as, nutrient deficiency (lack of carbohydrates), or adverse environment (low pH value, high temperature), which causes hydrolysis of peptidoglycan, a rigid membrane that surrounds the cytoplasmic membrane of most bacterial species. By controlling the lysis of LAB starter culture, it is possible to manage flavour and taste development and, at the same time, accelerate ripening period. As already mentioned, starter LAB culture produces intracellular lipases, peptidases and enzymes of amino acid catabolism (Lortal & Chapot-Chartier, 2005). The most complex and important process that occurs during cheese ripening is proteolysis, a breakdown of proteins into smaller peptides and amino acids. However, proteolysis also happens in milk before cheese production, and during the enzymatic coagulation. Proteolytic activity in milk is generally considered undesirable because it can lead to the degradation of milk proteins, particularly casein, and the development of off-flavours and odours. Proteolysis can occur due to the presence of proteolytic enzymes, such as plasmin, which is naturally present in raw milk, or due to the activity of microorganisms that produce proteases. Plasmin is a very resistant proteinase that causes formation of protease peptones leading to yield loss (Fox, 1989). Naturally present psychotropic bacteria are active during milk storage and with their extracellular proteinases can cause degradation of milk proteins. If proteolytic activity is not controlled, it can lead to significant changes in the quality of milk and milk products. For example, proteolysis can lead to the formation of bitter peptides, which can result in an unpleasant taste in milk and milk products. To prevent undesirable proteolysis in milk, various techniques can be used, including pasteurization, which can help inactivating proteolytic enzymes and reduce microbial activity, and the use of inhibitors such as lacto-peroxidase and protease inhibitors, which can help to prevent the breakdown of milk proteins. During

enzymatic coagulation, proteolysis occurs as a result of the action of proteolytic enzymes, including chymosin, and other proteases present in rennet, on the milk proteins. These enzymes break down the casein proteins into larger peptides, that later degrade to smaller peptides and amino acids, which contribute to the texture, flavour, and aroma of the cheese. The amount of rennet enzymes that remains in cheese curd depends on the type, temperature during manufacture and the pH at the whey drainage (Guinee and O'Brien, 2010). As the cheese ages, additional proteolysis occurs due to the action of enzymes present in cheese. Chymosin degrades b-casein making short hydrophobic peptides, which are bitter. Throughout cheese ripening, enzymes continue to break down the milk proteins. Proteinase and peptidase, present during ripening, originate from milk, coagulant, starter cultures (usually lactic acid bacteria), non-starter cultures, adjunct cultures (depending on a type of cheese, *e.g. Propionibacterium freudenreichii* in a production of Emmental cheese) (McSweeney, 2004). Lactocepin, the main LAB proteinase that is active during bacterial cell growth, its main role is to degrade caseins into medium size peptides that allow bacterial cells to advance. Other LAB proteinases are intracellular, such as, di- and tri-peptidases, oligoendopeptidases, aminopeptidases and proline-specific peptidases. NSLAB and adjunct culture proteinase systems are similar to those of LAB starter cultures. These enzymes break down the proteins further, leading to the formation of smaller peptides and amino acids, which contribute to the complex flavours and aromas of aged cheeses. Bacterial species require amino acids for their growth and reproduction, that is why they have a developed proteolytic system. The reason for proteolysis being the key process in cheese ripening lies in the following. During proteolysis, casein matrix hydrolysis occurs and softens the texture of cheese, formed amino groups and carboxylic acid change water binding properties hence causing the decrease in water activity (a_w) of the cheese. Furthermore, with the liberation of ammonia from amino acids, pH value of cheese matrix increases thus contributing to the activity of plasmin and the development of cheese texture. Released short peptides and amino acids have a direct effect on flavour of the cheese, but they also enter other reactions that form the flavour and aroma components of cheese. Obtained glutamic acid (Glu) is associated with the pleasant taste; isoleucine (Ile), lysine (Lys) and tyrosine (Tyr) bitter; proline (Pro) and lysine (Lys) sweet-bitter taste; alanine (Ala), glycine (Gly), serine (Ser) and threonine (Thr) sweet, while histidine (His) and aspartic acid (Asp) have a bitter-sour taste (Mikulec *et al.*, 2010). To summarize, indigenous milk proteolytic enzymes (*e.g.* plasmin) and residual coagulants in cheese matrix hydrolyse caseins to a wide array of large and medium-sized peptides who are further degraded to smaller peptides and free amino acids by starter LAB, NSLAB, and added adjunct culture. The extent and rate of proteolysis depend on various

factors, including the milk composition, type of cheese, the enzyme used, manufacturing process and the aging/ripening conditions. Cheese makers can control proteolysis by adjusting these factors to achieve the desired flavour and texture profile. During the process of cheese ripening, all mentioned components are gradually being converted into flavour and aroma molecules, at a slow rate because most cheese-ripening processes are carried out at low temperatures. The amount and proportions of individual amino acids and soluble peptides significantly affect the texture and organoleptic properties of cheese. Amino acid content is often used as an index of cheese maturity.

Following primary events, secondary biochemical changes are very important for the development of many volatile flavour compounds and include the metabolism of fatty acids and of amino acids. As mentioned before, free fatty acids take part in producing cheese flavour by acting as precursors for a series of reactions. Fatty acids are released through the process of lipolysis, which is the breakdown of milk fats in the cheese. The level of lipolysis and profile of free fatty acids are characteristic for a particular type of cheese. Once released, free fatty acids can be metabolized by various microorganisms present in the cheese. The most common fatty acid metabolism pathway in cheese involves the conversion of fatty acids into flavour and aroma compounds such as esters, lactones, ketones, and alcohols. This pathway starts with the oxidation of fatty acids by microbial enzymes, which produces Acyl-CoA molecules. These Acyl-CoA molecules are then further metabolized through a series of enzymatic reactions, resulting in the production of a range of flavour and aroma compounds. For example, butanoic acid is a fatty acid commonly found in cheese that can be metabolized by microorganisms to produce compounds such as diacetyl, which is responsible for the buttery aroma in some cheeses. Similarly, propanoic acid is another fatty acid commonly found in cheese that can be metabolized by microorganisms to produce compounds such as 2-methylpropanal, which is responsible for the nutty aroma in Swiss cheese. Free fatty acids react with ethanol, a product of lactose fermentation or amino acid catabolism, together making ethyl esters, a dominant ester found in cheese. Esters can also be synthesized by reaction of a glycerides and alcohol (ethanol). Flavour characteristics linked to esters found in cheese, such as, Gouda, Cheddar, Grana Padano, and Emmental cheese, are described as sweet, fruity and floral (Gao *et al.*, 2022). Esters can also contribute to the general aroma of cheese by reducing the bitterness and sharpness of free fatty acids and amines (Thierry *et al.*, 2017). Formed lactones, via intramolecular esterification of hydroxy fatty acids, are also given fruity attributes, similar to those found in apricot and peach, but also as sweet and buttery (Terpou *et al*, 2018). As esters and lactones are

the most important flavour components in Dutch-, Italian-, and Swiss-type of cheese, ketones are the predominant components in mould ripened cheese. Methyl ketones are found in Roquefort and Camembert cheese formed by the action of adjunct mould culture, and the rate of their production is not only affected by the physiological state of the mould, but also by temperature, pH value and the concentration of free fatty acids. Present ketones give specific strong musty and fruity odour to mould ripened cheese, but can also be found in Gouda, Gruyere, Edam, Parmesan, and Cheddar cheese. With the enzymatic reduction of methyl ketones, secondary alcohols are formed, and their conversion is favoured under the absence of oxygen. They give off wine, fruit and sweet like aromas to cheese. Present straight-chain aldehydes are described as grass like that at high concentrations can have an unpleasant odour. However, aldehydes are mostly considered as transitory compounds that are further being converted into other compounds (Thierry *et al.*, 2017). In addition to contributing to cheese flavour and aroma, fatty acid metabolism can also affect the texture of the cheese. For example, the metabolism of fatty acids can lead to the formation of free fatty acids, which can affect the pH and water-holding capacity of the cheese, leading to changes in texture. Overall, the metabolism of fatty acids is a complex and important process that plays a significant role in the development of cheese flavour and texture during ripening. Cheese makers must carefully control the ripening process to ensure that the metabolism of fatty acids occurs at an appropriate rate to produce desirable flavours and textures.

Amino acids are the building blocks of proteins, and during cheese ripening, proteins in the cheese are broken down into their constituent amino acids through the process of proteolysis. Once released, they can be taken down by several pathways generating aldehydes, alcohols, acids, esters, and sulphur compounds, which form the specific aromas of various types of cheese. First stage of amino acid catabolism is transamination, oxidative deamination, decarboxylation and desulphuration. Following the first, is the second stage converting α-ketoacids, amines, and amino acids into aldehydes, primarily by the action of deaminases on amines. And lastly, final stage of amino acid catabolism is aldehyde reduction or oxidation to alcohols and acids, respectively (Mcsweeney & Sousa, 2000). Transamination is initiated by microorganism aminotransferases that convert specific amino acids to corresponding α-ketoacid and, in the same time, synthesizing new amino acid via amino group transfer. Obtained ketoacids are further degraded by enzymes or chemical reactions into a range of compounds and molecules. Precursors in the aroma formation are amino acids with a branched chain (valine, isoleucine, leucine), aromatic acids (tryptophan, tyrosine, phenylalanine), and sulfur-

containing amino acids (cysteine, methionine) (Mikulec *et al.*, 2010). It is known that primary starter culture of bacteria dominates at the beginning of the technological process of cheese production and ripening. However, as the pH of the cheese decreases (action of LAB in the starter culture that ferments lactose into lactic acid) and the increased salt content (process of salting, brining, etc.), most of the lactic acid bacteria dies of, while the number of non-starter lactic acid bacteria increases during ripening and dominates at the end of the technological process of cheese production. The shift from starter culture dominance to non-starter bacterial dominance is a natural part of the cheese ripening process and is essential for the development of complex flavours and textures in the cheese. Following on from that, LAB and NSLAB aminotransferases most often use α-ketoglutarate to convert into glutamic acid that gives off umami taste, usually found in both Cheddar and Swiss cheeses. A number of attempts have been made to improve cheese flavour by adding α-ketoglutarate to cheese enhance aminotransferase activity and faster rates of amino acid catabolism (McSweeney, 2004). This is because α-ketoglutarate can act as a co-substrate for aminotransferases, providing an additional source of keto acid that can be used in the transamination reaction. In addition, α-ketoglutarate can also act as a signalling molecule, activating enzymes involved in amino acid metabolism and promoting the breakdown of amino acids during cheese ripening. Break-down of branched chain amino acids produces isovaleric (3-methylbutanoic acid) and isobutyric (2-methylpropanoic) acid that have sweet but also sour, bitter, rotten or buttery taste and smell, 2-methylbutane, 3-methylbutane, and 2-ethylpropane with a characteristic fruity or alcohol-like aroma, commonly found in Swiss-type cheese. However, aldehyde and alcohol products of branched-chain catabolism can cause defects in cheese (Mcsweeney & Sousa, 2000). The modification of aromatic amino acids produces phenylacetic acid (2-phenylethanoic acid), phenylacetaldehyde, benzaldehyde, cresol, phenol and indole, which have a floral or phenolic smell and are key components of the smell in hard and semi-hard cheeses. Less important, but still occurring, pathways in cheese are deamination and decarboxylation. Decomposition of aspartate by the action of aminotransferase produces oxaloacetate, which can be catabolized by further reactions to diacetyl, acetoin and 2,3-butanediol, which have aromas attributed to butter. Deamination is a pathway that involves the removal of the amine group ($-NH_2$) from the amino acid, producing ammonia and α-ketoacid. For example, the amino acid glutamate can be deaminated to produce α-ketoglutarate and ammonia. This is important in direction of generating nitrogen for the growth of microorganisms in the cheese. Ammonia is also flavour-related compound found in Camembert, Gruyère i Comté cheese (Mikulec *et al.*, 2010). With the accumulation of ammonia, there is an increase in the pH value of the cheese which may

have a stimulatory effect on the growth of secondary bacterial flora, but also it affects the firmness, causing mechanical resistance of the cheese to decrease. Amines can also be subjected to oxidative deamination leading to aldehyde production (Mcsweeney & Sousa, 2000). Decarboxylation involves the removal of the carboxyl group (-COOH) from the amino acid, producing a biogenic amine and carbon dioxide due to microbiological activity. For example, the amino acid histidine can be decarboxylated to produce histamine and carbon dioxide. For example, histidine may undergo decarboxylation, resulting in the formation of histamine and carbon dioxide. Biogenic amines exhibit biological activity as they serve as precursors in the biosynthesis of hormones, proteins, and nucleic acids, and they also contribute to the modulation of immune functions. Nevertheless, elevated levels of these compounds in cheese can exert toxicological effects upon consumption, manifested by symptoms such as hypertension, tachycardia, migraine, urticaria, and related disorders. In addition, biogenic amines influence both the quality and safety of cheese and may provoke diverse physiological responses in consumers. It is not exactly known why microorganisms form biogenic amines, but it is assumed that it is part of a defence mechanism in an unfavourable environment. Decarboxylation increases survival at low pH values because microorganisms introduce protons into the cell and expel amines and carbon dioxide thus establishing an optimal pH of the medium. Also, the formation can be a response to osmotic or oxidative stress, and even as one of the ways of obtaining ATP energy (Linares *et al.*, 2011). Bacterial decarboxylases show the highest activity at pH between 4.0 and 5.5, which is important considering the fact that pH value of cheeses is 4.5 for semi-hard, 5.1 – 5.4 for hard and extra hard cheeses at the end of the ripening period (Loizzo *et al.*, 2013).

Sulfuric compounds, such as, methanethiol, dimethyldi- and dimethyltri sulhide, methional (3-(methylsulfanyl) propanal), and thioesters, contribute to the cabbage, eggs, garlic and potatoe-like cheese taste and smell, are formed by the breakdown of the amino acids methionine and cysteine (Mikulec *et al.*, 2010). The mentioned aromatic components are found present in cheeses such as Camembert, Parmesan, and Swiss-type cheese. From the mentioned two, the most abundant amino acid in cheese is methionine which is found on casein remnants. Bacterial cells contain the enzyme methionine γ-lyase that can catalyze the methionine degradation reaction, however, this enzyme has not been identified in lactic acid bacteria but is believed to originate from secondary microflora or adjunct culture (Jelovac *et al.* 2011). Other than bacterial lysis, methionine breakdown and the formation of specific odours and tasteful compounds is affected also by redox potential, transport of precursors, cation accumulation and

the location of intracellular catabolite enzymes (Bonnarme *et al.,* 2001). Another reaction that occurs during cheese ripening is racemization. This pathway involves the conversion of a D-amino acid to an L-amino acid or vice versa. For example, the amino acid aspartic acid can be racemized to produce a mixture of D- and L-aspartic acid. This pathway can affect the flavour and texture of the cheese by changing the interactions between amino acids and other components in the cheese. Overall, the metabolism of free amino acids during cheese ripening is a complex process that involves multiple pathways, as shown in **Figure 1**, and can have a significant impact on the flavour, texture, and nutritional properties of the cheese.

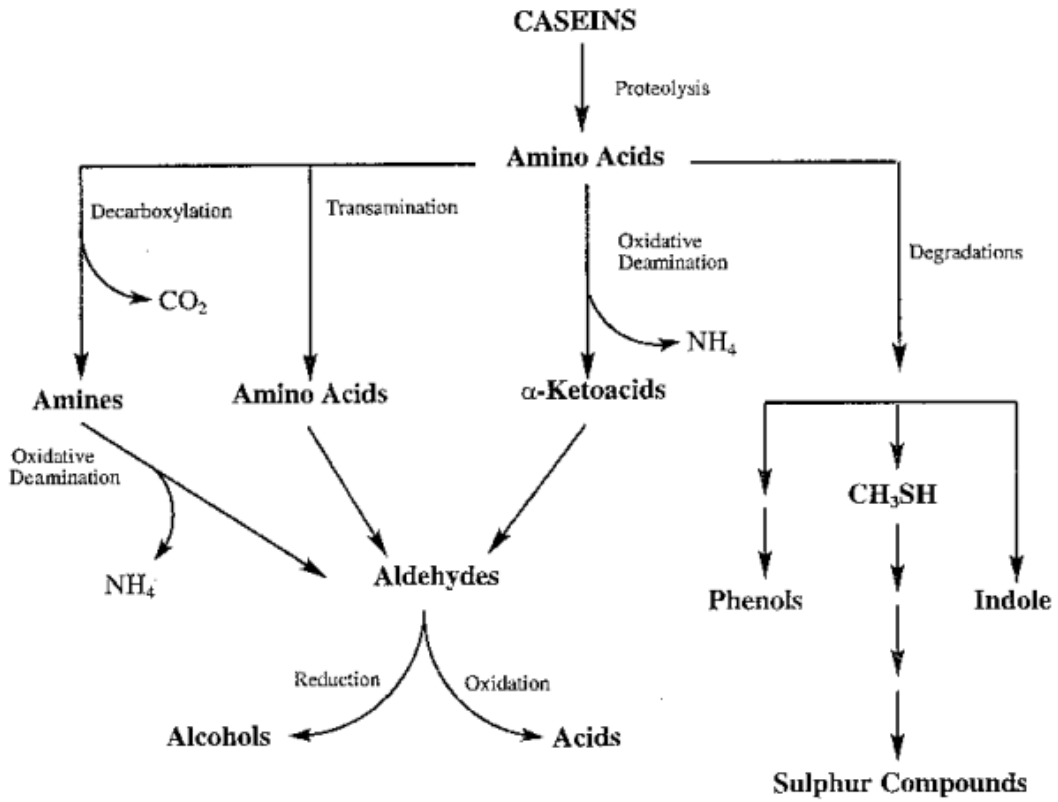


Figure 1. General pathways for the catabolism of Free Amino Acids (FAA) (Mcsweeney & Sousa, 2000)

In addition, during cheese ripening, redox reactions can occur. These reactions involve the transfer of electrons between molecules that can lead to the formation of colour compounds, such as yellow and red pigments. However, a well-known additive in cheese production is annatto dye (β-carotene), which is added to milk for cheese production in order to achieve uniformity and improve the colour of the cheese, it is commonly added during the production of Cheddar cheese.

As already established earlier, depending on all the procedures that precede ripening and during ripening, cheese with certain characteristics will be formed. For many cheese types the exact result of the ripening is unpredictable. That is why, at this point, is important to establish adequate ripening parameters and take proper care of the cheese during maturation. After the salting process, the cheese surface is still wet, and it is important to air dry freshly formed cheese in order to remove moisture. It should be conducted at the temperature between 17 – 19°C and relative humidity of 75 – 85 % with sufficient air flow for a few days. In case of excess moisture on the cheese surface, undesirable microorganisms can develop, such as, for example, yeasts on the surface of cheeses with an intact rind (without desirable growth of microorganisms on the surface), or undesirable moulds in mould-type cheese that inhibit the growth and activity of the added and desirable mould varieties. Yeasts act as deacidifiers by breaking down the lactic acid on the surface of the cheese and thereby increasing the pH value of the surface of the cheese. This is desirable in the production of smear-ripened cheese because the growth and metabolic activity of the yeasts create a sticky coating on the surface, and also generates substances that stimulate the growth of surface microflora which contributes to the taste and flavour of the cheese. Furthermore, it is also important to dry the surface of the cheese before applying films or coatings, which prevents good adhesion, increases peeling and slime formation as an increase in the activity of bacteria on the surface of the cheese. Paraffin, fungicides, food colours, and other different dispersions can be used as coatings.

Cheese ripening rooms or chambers are the final destination before the cheese packaging, transport and distribution. In the past, ripening rooms were placed underground, which ensured a constant temperature and humidity. Nowadays, industrial ripening rooms are made of materials that provide good insulation, are easy to clean, disinfect and maintain, and are equipped with devices that enable space conditioning. The most important parameters are temperature, humidity and air flow. The air conditioning system consists of a fan (air transfer), a heat exchanger (heating and cooling), a device for air purification and humidification, a space for combining two streams of air with different properties, and a controlled system for automatic temperature and humidity control. The ripening parameters change as the maturation of cheese extends, so it is important to adapt the conditions to the degree of cheese maturity. Ripening temperature for most cheese varieties is between 10 and 15°C. Higher temperatures are causing faster biochemical changes during ripening (enhanced proteolysis) hence the faster aging of cheese which can diminish the durability of the product and its quality. On the other hand, lower temperatures are slowing down the maturation process, which is necessary not a

bad thing, but it can retard specific aroma and flavour development changes due to limited microbial activity. With certain types of cheese, it is necessary to carry out different temperature treatments during ripening. For example, during the ripening of Swiss-type cheese (Emmentaler), after the initial ripening temperature of 10 to 15°C, the temperature is increased to 20 to 24°C, for a certain period of time (usually 6 weeks), in order to ensure the optimal course of propionic fermentation, which releases carbon dioxide thus creating characteristic cheese eyes (Bachmann, 2011). Ripening can also be carried out at temperatures lower than 10°C, which is characteristic in the production of Cheddar cheese, which is why the cheese ripens for 9 to 12 months at a temperature of 4 – 6°C. The reason for the low temperatures is the already intense activity and the peculiar development of the aroma by the action of the starter culture. Cheeses with blue (Gorgonzola) and white (Camembert) moulds also ripen at lower temperatures due to their strong proteolytic and lipolytic activity. Excessive proteolysis in such cheeses causes too soft cheese texture and bitter taste, and lipolysis results in a dull and off-taste and smell.

The ripening of the cheese is also a process that involves exchanging substances and energy with the environment. In that sense, cheese with time releases heat as a result of water evaporation from the cheese surface. There is an increase in temperature and moisture content in ripening rooms, consequently promoting loss and defect during maturation. Increased moisture causes the development of yeasts on the surface of the cheese, which is why moisture regulation is an important ripening parameter. Relative humidity (RH) ranges from 75 to 95% at temperature between 10 to 13°C, for a colder storage, relative humidity is lower (60 – 65%). This is because at lower temperatures cheese loses less moisture, and the balance between released and absorbed moisture is the result of constant cheese weight. Excessive temperature and humidity can cause the cheese to "sweat" therefore moulds cannot grasp onto the surface, which is not beneficial in the production of the smear-ripened cheese, thus undesirable characteristics of the cheese may develop. On the other hand, too low temperature, humidity and too strong air flow cause dehydration and weight loss of the cheese, as a result of which production efficiency is low. The higher the temperature and humidity in the ripening rooms, the greater the growth and development of yeasts (Leclercq-Perlat *et al.*, 2015), which is undesirable in the ripening of hard and semi-hard cheeses such as Emmentaler, Gouda and Tilzit, and desirable in the maturation of smear-ripened cheese, like Limburger or Romadur. However, by applying low humidity during cheese ripening, the surface hardens, during which the migration of moisture from the inside of the cheese is prevented, which can cause losses

during ripening. With the inadequate and low relative humidity of the ripening chambers, the cheese surface is too dry and dehydrated, immersing hard cheese rind and the inability to develop surface microflora of the cheese. In order to prevent temperature and humidity retention over and between the cheeses on the shelves, it is important to ensure air flow. Depending on the degree of maturity of the cheese, the air flow should be between 6 – 18 cm/sec. The flow and inflow of fresh air is also required during the formation of different gases. During cheese ripening, a lot of gaseous substances, such as ammonia, carbon dioxide (carbon dioxide) and oxygen (O_2), are formed and released. Ammonia, in too much concentration, is particularly unwanted because it causes a reduction in the quality of the cheese and adverse work conditions due to sharp and unpleasant odours, irritation, and potential toxicity. This is especially prominent in mould- and smear-ripened cheeses, or in ripening rooms that are overcrowded with cheese. Cheese maturation in excess ammonia shows an increase in pH value, rapid protein break-down and retarded activity of surface microflora, hence the faster maturation period and undesirable textural (closed cheese structure) and flavour (bitter, sharp) changes.

Shelves in ripening rooms/chambers can be made of wood, plastic or stainless steel. Wooden shelves are prone to the development of a secondary microflora consisting of different types of yeasts and bacteria because of its ability to retain moisture. This necessarily does not have to be a negative thing. With adequate maintenance it is possible to promote the development of a particular microflora that can ultimately give cheese desired characteristics and protect it from the pathogens. This is seen as an advantage in comparison with plastic or metal shelves, however, they are easier to clean, dry, and disinfect. Regardless of the appropriate hygiene of the shelves, cheese must be turned over to ensure uniform and even ripening, prevent build-up of moisture, and to retain shape. This is especially important for mould- and smear-ripened cheeses to ensure even development of microflora. Depending on the size of the cheese, the turning interval is different, so the cheese of smaller dimensions is turned over every two days, and the larger ones from 3 to 4 days. In large industrial plants, this procedure is automatically performed because it requires a lot of time and work. Along with turning, washing and brushing of the cheese surface once to three times a week is performed. The cheese is washed with clean water, sometimes with addition of salt, to remove unwanted yeasts and moulds (this step is not carried out with mould- and smear-ripened cheese), and to prevent the dehydration of the cheese rind (Gregurek, 2015).

Not all cheeses mature in ripening rooms, a lot of them ripen in brine, whey, oil, wine, and even in vinegar. The ripening of cheese in brine refers to the process whereby cheese is

soaked in a saltwater solution called brine to mature and develop flavour. Brine, which is a solution of water, salt (10 – 15 % of NaCl), and sometimes other additives, is used as a medium for ripening certain types of, so called, white cheese, such as Feta, Domati, and some varieties of Blue cheese. During the ripening process, enzymes naturally present in the cheese break down proteins and fats, leading to changes in the texture, flavour, and aroma of the cheese. The brine aids in this process by providing a controlled environment that allows the cheese to mature uniformly without drying out or developing mould. The high salt content in the brine acts as a preservative to inhibit the growth of unwanted bacteria, creating a desirable environment for the beneficial bacteria that contribute to the unique cheese characteristics. Cheeses that are ripened in brine are typically firm white in colour without cheese rind and have a slightly salty, tangy flavour. The ripening process of cheeses in brine is controlled by maintaining the temperature and concentration of the brine. Ripening time is longer if the temperature is below 9°C and may take from 3 to 6 months, but if the temperature is between 9 – 16°C cheese can be ready for consumption in about one month. The amount of time that cheese spends in the brine can vary depending on the type and size of cheese and the desired level of ripeness. Some cheeses may only need to be soaked in brine for a few days, while others may require several months or even years of aging to fully develop their distinct flavour and texture.

As already emphasized, the duration of ripening time is different for different types of cheese. In general, soft and fresh cheeses (such as ricotta, feta, and mozzarella) have a shorter ripening time, usually ranging from a few days to a few weeks. These cheeses are typically consumed soon after production and are not aged extensively. Semi-hard cheeses (such as Cheddar, Gouda and Swiss-type cheese) have a longer ripening period, usually ranging from a few months to a year. During this time, the cheese develops a firmer texture and a sharper flavour. And finally, hard cheeses (such as Parmesan, Pecorino and aged Cheddar) have the longest ripening period, often taking a year or more to reach their desired flavour and texture. The aging process for hard cheeses involves the slow breakdown of proteins and fats, resulting in a crumbly texture and complex, nutty flavour. Hard cheeses contain a high amount of dry matter which inhibits enzymatic activity and consequently leads to extended ripening time. Overall, the length of the ripening process will depend on the specific cheese variety and the desired flavour and texture characteristics, which is shown in **Table 1**. Longer ripening times can result in more complex flavours and desired textural characteristics, making it an important aspect of cheese production.

Table 1. Most common cheese types, their ripening period and characteristics

Cheese type	Ripening period	Specific characteristics	Key production step
Camembert	3-4 weeks	Soft, creamy, distinct earthy flavour	*Penicillium camemberti* inoculation
Brie	1-2 months	Soft, creamy, mild, buttery flavour	
Feta	2-3 months	Soft, crumbly, tangy, salty flavour	Brining
Swiss-type cheese (Emmental)	2-4 months	Firm, nutty, sweet flavour with characteristic holes	Addition of propionic bacteria - formation of cheese eyes
Roquefort	3-9 months	Veined, creamy, sharp, pungent flavour	*Penicillium roqueforti* inoculation
Gouda	2-12 months	Semi-hard, creamy, nutty, sweet flavour	Curd washing
Manchego	3-12 months	Hard, nutty, buttery flavour with distinctive crisscross pattern on rind	Curd cutting, from 5 to 10 mm
Cheddar	3 months - 2 years	Hard, sharp, tangy, nutty flavour	Cheddaring
Parmesan	1-2 years	Hard, granular, nutty, salty flavour	Curd cooking

Small changes and oscillations in cheese production can have a big impact on the final appearance, taste and aroma of the cheese. The inoculation of the specific microbial cultures is a crucial step because they carry out essential metabolic activities that cause differences in the development of aroma and texture during ripening. For example, in the production of Roquefort cheese, culture containing *Penicillium roqueforti* is inoculated in milk, afterwards cheese curds are pierced with needles to introduce the mould spores throughout the cheese. *Penicillium roqueforti* grows and develops blue veins as it breaks down the proteins and fats. In Gouda cheese production, starter cultures are primarily used to initiate the fermentation process. Lactic acid bacteria, such as *Lactococcus lactis* and *Streptococcus thermophilus*, are inoculated into the milk to convert lactose into lactic acid. These cultures contribute to the development of

flavor, acidity, and texture in Gouda cheese. Formation of cheese eyes, caused by inoculated propionic acid bacteria, is characteristic for Swiss-type cheese, such as Emmental cheese. Mentioned bacteria in cheese metabolizes lactose and releases carbon dioxide, among other by-products, that is trapped and accumulates in the cheese curd air pockets and forms gas bubbles, commonly referred as cheese eyes, which are visible on the cheese cross-section. Other determining steps include milk selection (milk with a higher fat content tends to produce a slower-ripening cheese), coagulation (gentle coagulation can lead to a longer ripening period and a more complex flavour), curd treatment (curd cutting - retaining more moisture causes faster ripening), and salting (retardation of microbial activity – extended ripening time).

2.2. Traditional Cheese Ripening

Traditional cheeses were created spontaneously as a result of the interaction between a man and the environment and are characterized by a strong connection with the area where they were produced and the influence of the culture and history of the people from that area. In general, traditional cheeses are made from thermally untreated milk, with or without the use of dairy culture and natural rennet, and with a lot of manual work. Cheeses manufactured in the traditional way have a specific, unique taste, smell, aroma and texture, which cannot be fully reproduced in industrial conditions. Cheese made from raw milk ripen for a shorter period of time, have a more pronounced taste and a different texture, which are the result of intense and uncontrolled biochemical reactions during cheese ripening. The biochemical processes of proteolysis and lipolysis are considered the most significant parameters in the identification of the origin of traditional cheeses produced from raw milk (Božanić, 2015). Traditional methods of cheese ripening vary depending on the type of cheese, the region, and the specific techniques used by individual cheese makers. However, some general principles apply to many traditional cheese-ripening methods.

One common method of cheese ripening is to place the cheese in a cool, humid environment, such as a cellar or a cave. The temperature and humidity cannot be fully controlled to promote the growth of specific microorganisms and enzymes that contribute to the ripening process. In some cases, cheese makers may use specific microorganisms, such as moulds or bacteria, to promote the development of certain flavours and textures in the cheese. Ripening of cheese in a cave is a traditional method that has been used for centuries. It involves storing unprocessed cheese in a natural cave environment where certain microbes, temperature, and humidity levels that aid in the development of the cheese's flavour, aroma, and texture. The process starts by preparing the cheese curds and then placing them in the cave to allow for

controlled aging over a period of time. The length of time the cheese spends in the cave depends on the type of cheese being made and wanted sensory characteristics. The unique features of the cave environment play a critical role in the ripening process. The cool and humid conditions, estimated around 15 – 25°C and 85 – 90% respectively, inside the cave provide the ideal environment for the growth of wild microflora that contributes to the cheese's flavour and texture. The humidity also helps to prevent the cheese from drying out and developing unwanted aromas. Such conditions promote the development of different types of fungi that grow naturally on cave walls and later colonize cheese during its ripening stage. Their enzymatic proteolytic and lipolytic activity results in changes in the cheese's texture, making it softer, creamier, and develops distinctive strong, yet sweet, creamy and earthy taste. Among ripening beneficial microbiota, toxigenic species are also present possibly contaminating and affecting the safety of cheese. Nevertheless, such cheeses are still considered a specialty around the world characterized by good level of quality, and accordingly, a higher price. Cheese care involves turning over the cheeses, and at the end of the ripening period they are brushed and washed to remove visible moulds that could have a negative effect on consumer preferences (Anelli *et al.*, 2018).

Another traditional method of cheese ripening involves aging the cheese in a natural casing, such as animals' stomach or skin. The natural casing can help to regulate the moisture and airflow around the cheese, which can promote even ripening and the development of desirable flavours and textures. Cheese in a sack was initially produced in Mediterranean area from raw ewe's milk, but a variation of cows and goat milk cheese is also present. After the wool shaving, fat removal, washing and drying, ewe's sack is soaked in whey or warm water to soften, and additionally disinfected with brandy or vinegar. Freshly made cheese curd is then cut and placed with salt in sack with a direct skin contact. Animal skin is made of fibrous proteins (e.g. collagen, elastin, etc.) whose composition affects the permeability of the sack in terms of air and moisture, thus various chemical and biochemical processes take place. Other than that, sack is naturally rich in secondary microflora consisting of moulds and yeasts. It is important to ensure anaerobic conditions by filling and stuffing the curd into sack that must not have any holes and damage that would allow air inlet. Under such ripening conditions for 2 to 3 months, cheese undergoes intensive lipolysis and proteolysis assisted by indigenous milk and skin microflora. Generally, cheese from sheep's milk has intense aroma and flavour, but with mentioned ripening environment the result is strong odour, piquant flavour and aroma, and spicy-salty taste. The texture of cheese is smooth with specific cheese granules (Tudor Kalit *et*

al. 2020). In some traditional cheese-making cultures, cheese is also ripened by burying it in the ground (anaerobic fermentation) or wrapping it in leaves or other natural materials. These methods can provide a unique flavour and aroma to the cheese and can help to regulate the temperature and humidity during the ripening process. When cheese is wrapped in chestnut, grape or fig leaves, it creates a microclimate inside the wrapping that encourages the growth of specific moulds and bacteria. These microorganisms help to break down the cheese and transform its texture and flavour to earthy, nutty, fruity and/or floral over time. The use of traditional equipment that has been passed down over the generations and used during cheese manufacture can have an effect on the final product characteristics. For example, pasteurization and curdling of milk in copper vats leave metal residues that can give specific aroma to cheese during ripening. Wooden milk sampling buckets, spatulas, sticks, moulds or shelves have a complex biodiversity of microorganisms secured on the surface in a form of biofilm that interacts with milk and/or cheese providing specific taste and quality (Papademas & Bintsis, 2017).

Overall, traditional methods of cheese ripening are often labour-intensive and time-consuming, making a considerable expense for small-scale producers. Likelihood of spoilage, inconsistent quality, and limited shelf life are another risks with natural cheese ripening process. However, traditional cheese ripening allows for the development of unique and complex flavours that cannot be replicated by other means such as artificial additives or shortcuts. Traditional cheese-making methods often use locally sourced milk and rely on traditional techniques that avoid the use of synthetic chemicals, making them more environmentally sustainable. And finally, traditional cheese-making is often steeped in cultural heritage and traditions, making it an important part of cultural identity and pride. For that reasons, many cheese makers continue to use these traditional methods today, often in combination with modern techniques, to produce high-quality, artisanal cheeses. As already mentioned, traditional cheese ripening involves natural processes that occur without advanced technology or equipment. It is typically done in small batches of cheese, often made by artisanal cheesemakers. The process can take months or even years, during which time the cheese will be monitored and cared for by hand. Industrial cheese ripening, on the other hand, is a more standardized and automated process. It is typically used in large-scale cheese production, where consistency and efficiency are key. Industrial cheese makers use specialized equipment and technology to control the temperature, humidity, and ventilation of their cheese rooms, aiming to achieve a consistent and a safe product.

2.3. New Achievements in Cheese Ripening

The basis of the cheese production process over the years has not significantly changed, but the production of larger quantities of cheese, of uniform quality and safety for consumers, has been made possible. The most common problems in cheese production are the lack of knowledge and technology, which is extremely important in order for cheese producers to be competitive on the market and thereby increase the volume of their production. One of the challenges is the ripening time of the cheese, which is known to be the longest process in its production. The ripening period is different for individual cheeses, and for semi-hard cheeses, in most cases, it lasts from two to six months. Ripening is a complex biochemical process, and any improvement with the possibility of practical application can have economic significance for producers.

Wrapping the cheese in foil is one of the ways to preserve the cheese and reduce the physical labour involved in its care. Once the cheese is formed and salted, it is wrapped in a thin layer of plastic foil, which helps in regulating the levels of moisture and oxygen that come into contact with the cheese, but in the same time allowing for the controlled release of carbon dioxide and other gases that are produced by the ripening process. It prevents cheese from drying out and physical damage (cracks), while also limits the development of bacteria, moulds, and mites on its surface preserving the flavour and aroma of the cheese, and preventing unwanted flavours from developing. On the other side, foil can limit air circulation, prevent development of unique flavour, and/or cross-contamination from the used foil to the cheese can occur, all of which may reduce the sensory quality of the product. It was established that plastic films affect the basic microflora and that the number of lactic acid bacteria is higher in cheeses in foil, during the entire ripening period, and in cheeses without foil, the number of micrococci is much higher. These results also explain the differences in the taste and smell of these two groups of cheeses. Cheeses without foil have a stronger taste and smell, as a result of the proteolytic and lipolytic activity of micrococci, while cheeses in foil have a milder taste and smell, because lactic acid bacteria are not pronounced proteolytic or lipolytic (Šutić *et al.* 1980). Apart from plastic foils, cheese can be protected by various coatings/films. Edible packaging presents an intriguing option for packaging food that is distinct from traditional methods. This type of packaging consists of a thin layer of edible material on the food surface and provides comparable protective qualities. Various biopolymers such as polysaccharides, proteins and lipids are used for the production of edible films, and certain physical, mechanical and barrier properties depend on the material from which they are made. The characteristic that makes them

stand out is the possibility of enrichment, which enables more functional properties than classic packaging. Thus, depending on the raw material used, the antimicrobial action of the film, the prevention of oxidation, improvement of sensory and nutritional properties can be emphasized. Advantage of these materials on cheese during ripening can be seen in advanced protection, as edible coating creates a barrier between the cheese and outside environment, thus protecting the cheese from unwanted microorganisms and contaminants, and prolonged shelf life. Furthermore, similar to plastic foil, edible coating can help reduce moisture loss, one of the major problems in cheese ripening. But the most important is its contribution towards flavour development and texture maintenance. Some studies have shown that certain types of coatings, such as those made from chitosan or whey protein, can enhance the flavour and aroma of cheese during ripening. Other coatings, such as those made from starch or cellulose, may have a more neutral effect on taste and flavour. In addition to the mentioned materials used as a base, different plant extracts, for example from carob and eucalyptus, can be added, which at the same time have good thickening and stabilizing properties, and antioxidant or antimicrobial properties, which can prevent and/or slow down unwanted reactions on the cheese surface. And lastly, due to the possibility of their consumption they contribute to the reduction of accumulated waste as they are biodegradable (Bourbon *et al.*, 2011). Disadvantages of using edible coatings can be seen in higher production expense (added cost of producing and applying the coating makes the cheese more expensive), off-taste (overpowering of the edible coating flavour), unwanted texture changes (altering cheese texture), and limited use as edible coatings can be used only or certain varieties of cheese.

Various additives and techniques are used in the production of cheese in order to increase its safety and speed up its ripening period. The addition of exogenous enzymes is one of the ways cheese makers can accelerate the ripening process by breaking down some of the cheese components faster than they would naturally. A mixture of enzymes (proteinases, peptidases, lipases) can be added in free form or encapsulated to ensure controlled release during cheese maturation causing acceleration of, not one, but multiple ripening steps. The same thing can be achieved with the addition of attenuated starters, consisting of LAB cells, that are unable to ferment lactose into lactic acid, but are rich with wide range of enzymes. Such form of starter is achieved through thermal treatments (heat-shock, freezing/thawing), spray-, freeze-drying or by the genetic modification causing lactase-negative mutants, ultimately resulting in "weakened" cultures. Although it has shown a lot of potential in accelerating the ripening of cheese, genetic modification is a sensitive concept in the eyes of consumers, which is why such

products have little chance of success on the market. Acceleration of ripening can be achieved by increased rate of bacterial cell lysis with the use of lysozyme. The same enzyme can be also used as a preservative since it has been successful in the prevention of late gas blowing commonly recurring in ripening of hard and semi-hard cheese. However, the rate of lysed bacterial cells should be carefully monitored to ensure that the cheese is safe for consumption and meets regulatory standards. As mentioned before, the role of adjunct cultures is to improve cheese flavour and to advance cheese maturation. Naturally, they don't have the ability to break down carbohydrates, but they are characterized by high proteolytic and lipolytic activity. That is why most cheese varieties with adjunct cultures, such as mould-, smear-ripened cheese, etc., have an intense flavour and a soft, creamy texture (McSweeney, 2007).

Modification of ripening parameters is probably the simplest and safest way to control cheese ripening. Elevated ripening temperature accelerates protein and lipid degradation reactions, and affects the activity of cheese microflora. However, extensive temperature ripening regime can lead to negative reactions in regards to flavour, aroma and texture development. It can cause microbial spoilage due to a change in the dynamics of dominant bacteria (McSweeney, 2007).

The researchers saw how the application of certain ripening conditions can make it easier to solve microbiological problems and maintain hygiene during cheese ripening. Ozone (O_3) is a powerful oxidant that is commonly used in various industries, including dairy industry, for its highly effective disinfectant properties. It is a natural gas generally recognized as safe and is therefore becoming widely accepted in the food context as a cost-effective and environmentally friendly preservative because it does not produce any harmful residues or by-products. Ozone is beneficial in cheese production because it improves the safety, quality, and shelf life of cheese. In low concentrations and short contact time, ozone acts as an effective antimicrobial agent. Precisely because of its oxidation capacity, ozone inactivates microorganisms (bacteria, viruses, and fungi) by progressive oxidation of cellular components (Jin-Gab *et al.*, 1999). Various hazards such as, for example, the development of pathogenic microorganisms on the surface of cheese or presence of moulds in the air of ripening rooms/chambers or in the storage rooms can be easily solved by applying ozone gas, where its effect has been proven (Varga & Szigeti, 2016). In addition to its antimicrobial properties, ozone can also effect the texture and flavour of cheese. When in contact with cheese, ozone oxidizes organic substances which results in protein break-down, fat aggregation and enzyme inactivation (Pandiselvam *et al.*, 2022). In addition to sensory properties, ozone not only

prevents discoloration, it also has the ability to bleach out some of the pigments that are present in cheese, resulting in a brighter and more attractive product. Depending on the concentration used, it can lead to improved or unwanted flavour and texture changes. However, food with higher fat content requires higher ozone concentrations in comparison to low-fat food (McHugh, 2015). Generally, concentrations above 2.0 ppm for a short contact time are used for inactivation of bacteria activity. Nonetheless, even lower concentrations, from 0.2 – 0.3 ppm, have shown a decrease of mould-covered areas on cheese surfaces. Sensory properties of cheese were not altered even at the concentration as high as 10 ppm, showing the beneficial effect of ozone (Varga & Szigeti, 2016). There is no requirement in constantly maintaining ozone levels in the ripening rooms, as seen in the many researches where periodical ozonisation managed to prevent microbial growth on cheese and prolonged its shelf life (Gabriel'yants' *et al.*, 1980; Shiler *et al*., 1983). Cheese that has been treated with ozone has been found to remain fresh and flavourful for a longer period of time, reducing waste and increasing profitability for producers.

Furthermore, the application of modified atmosphere in cheese production also has a positive effect. The modified atmosphere includes the application of nitrogen (N_2) and carbon dioxide gases in different concentrations, which have proven to be grateful collaborators in the fight against microbial growth, chemical degradation and preservation of the desired characteristics of taste, smell, appearance and texture of cheese. Different ratios of mentioned neutral gases can be applied to cheeses. Usually, concentration of gases is calculated in liters per kilogram of cheese. Concentration of around 75 – 80% nitrogen and 25 – 20% carbon dioxide has produced cheese with good overall quality and microbiology. However, cheese is a living ecosystem that is constantly changing. Carbon dioxide dissolves in water, but also in lipids, it can be consumed by anaerobic bacteria or produced by aerobic or anaerobic microorganisms. As the ripening temperature increases, the concentration of carbon dioxide also increases as a result of increased exchange of gases between the cheese and the environment, known as cheese respiration. In other words, its applied gas concentration is constantly changing. It is anticipated that the presence of carbon dioxide will lead to a decline in pH of cheese that is believed to be linked to the creation of carbonic acid, acidic amino acids, and the generation of free fatty acids while undergoing proteolysis and lipolysis. However, as proteolysis occurs and amines and ammonia are formed (when carbon dioxide accounts for 50% or less), there is an increase of pH value during the ripening process, predominantly found in surface-ripened cheeses. Changes in textural and sensorial properties were shown in a research on semi hard cheese packaged in a different concentrations of carbon dioxide and nitrogen,

where cheese exposed to 100% of carbon dioxide resulted in drying-out of cheese that had dry and crumbly texture, rancid flavour and taste and a darker colour rind (Juric *et al*., 2003). Alternating the ratio of carbon dioxide and N_2 in favour of nitrogen, flavour deficits caused by the sole use of carbon dioxide, undesired carbonated or "bubbly" flavour can be eliminated while maintaining freshness and controlling the microbial population of cheese (Khoshgozaran *et al*., 2012). A research was conducted on a Dutch-type cheese where initial concentration of 40% carbone dioxide and 60% N_2 was inserted into cheese packaging. After 60 days of ripening, the ratio of carbon dioxide decreased causing the number of facultative anaerobes, such as yeasts and moulds to increase, and in contrast number of coliform microorganisms decreased until the ripening time of 90 days. This could be explained by the created favorable environment in the absence of oxygen and reduced carbone dioxide concentration. Such packaging conditions also extended the cheese's shelf life (Panfil-Kuncewicz *et al*., 2014). The use of modified atmosphere in cheese packaging is widespread and tested, where it has been proven to improve the appearance of the product, prevent microbial growth and chemical degradation, preserve the desired sensory characteristics (colour, taste, smell and texture) and extend the cheese's shelf life (Atallah *et al*., 2021). However, the application of the aforementioned mixture of gases has not yet been tested in ripening cheeses during cheese ripening.

Among the various non-thermal preservation methods employed in the food industry, the use of high-pressure processing in cheese manufacture has proven to be effective. The application of elevated pressure during the ripening phase offers the advantage of reducing or inactivating undesirable microbial populations while preserving the cheese's physical and chemical properties. Ripening under all the aforementioned conditions can have a beneficial effect on shortening the process itself, which is of economic importance for every producer, and which consequently leads to an increase in their production, that is, competitiveness on the market.

2.4. High-pressure

High pressure processing is a non-thermal food preservation technique that utilizes high pressure, greater than atmospheric, to enhance and preserve food quality and safety. It involves exposing food to high pressure anywhere from 1 – 10 mbar for several weeks and months, up to 600 MPa for a few minutes to hours. The high pressure has shown to be effective in the inactivation of microorganisms like bacteria, moulds, yeasts, viruses and parasites, causing significant damage to their cellular structures, such as cell walls, membranes, and intercellular

proteins. When food is subjected to high pressure, the pressure applied is uniformly distributed throughout the food product, causing the cells of microorganisms to compress and rupture. This process damages the enzymes and proteins necessary for the microorganisms to survive and reproduce, effectively killing them. Additionally, high pressure can also cause changes in the physical and chemical properties of the food, such as modifying the pH, water activity, and oxygen levels, which further inhibits the growth and survival of microorganisms. The combination of these effects makes high pressure an effective method for reducing the microbial load in food, improving its safety and shelf life. Milk naturally contains antimicrobial compounds that act as a protective medium but are heat sensitive. By applying high pressure on milk in exchange for pasteurization, it is possible to influence the inhibition of the pathogenic microorganisms' development, such as *Listeria monocytogenes*, where antimicrobial compounds of the milk and pressure treatment act synergistically to enhance bacterial inactivation (Styles *et al.,* 1991). Research conducted on the microorganisms inoculated into ovine milk showed that gram-negative microorganisms (*Pseudomonas fluorescens*, *Escherichia coli*) were more sensitive to pressure than gram-positive ones (*Listeria innocua*, *Lactobacillus helveticus*, *Staphylococcus aureus*) (Gervilla *et al.,* 2000). This can be explained due to the fact that gram-negative bacteria have thinner cell wall, their membrane is more fluid and contains lipopolysaccharides that can be easily disrupted by pressure. On the other hand, gram-positive bacteria have developed various resistance mechanisms to survive extreme conditions. Pressure inactivation is not only applicable to the vegetative form of cells, but also to bacterial spores. High pressure can cause the spores to break apart and lose their ability to germinate and grow, effectively killing them. However, it is important to note that not all spores are equally susceptible to high pressure inactivation, and some may require higher pressure or longer exposure time to achieve the desired effect. Not only does high pressure treatment affect food microflora, it also causes direct or indirect changes in their properties. It preserves food properties, including taste, texture, and nutritional value, without the need for artificial preservatives. This is due to the fact that high pressure does not involve heat treatment, which can damage nutrients and alter the quality of food. However, high pressure does affect larger molecules with non-covalent bonds, like polysaccharides, proteins, enzymes and nucleic acids, that cause changes in food products. The activity of enzymes, changes in the conformation of enzyme substrates, and enzyme availability is also largely affected by the high pressure treatments. Consequently, the milk coagulation process and cheese making properties of the milk are indirectly impacted by the high pressure treatment due to the changes in milk proteins, which includes casein molecules reduction and β-lactoglobulin denaturation (O'Reilly *et al*.,

2001). A number of studies reported that applied high pressure between 200 – 500 MPa decreases rennet coagulation time, in other words, curd formation is faster. Changes in the rate of coagulation could potentially cause changes in gel structure, thereby leading to changes in cheese composition. As a result, the quality and ripening properties of the cheese could also be impacted. Furthermore, denaturation of whey proteins, caused by the application of pressure, followed by the rennet coagulation of caseins, may contribute to increased cheese yield. Through this sequence of events, whey proteins are incorporated into the protein network, and together with the increased amount of moisture, they affect the higher yield of cheese. The retention of moisture can be explained by the fact that aggregated casein micelles and fat globules in high pressure treated milk are not that closely connected as in non-treated milk thus leaving enough space for moisture to be trapped. Not only that, but the formed curd has a finer gel network that reduces whey syneresis. All the mentioned changes ultimately affect cheese sensory quality and consumer acceptability. As aforementioned in milk, microorganisms in cheese are also largely affected by the high pressure treatment.

Cheese is considered to be a safe dairy product. Its acidic nature further impeded by organic acids, inhibitory compounds and a low water activity are significant obstacles for pathogenic or spoilage microorganisms to thrive. However, additional tools, such as high pressure processing may be necessary to ensure the microbiological safety of cheese varieties with specific physicochemical or microbiological characteristics. For example, cheese made from raw milk is considered to be hazardous, especially for vulnerable populations, such as individuals with low immunity, pregnant women, elderly, children, etc., due to the absence of thermal processing of milk that eliminates pathogens. Most common pathogens found in raw, unprocessed milk are from the bacterial family *Enterobacteriaceae*. Rodriguez *et al.* (2005) conducted a research on the influence of high pressure treatment on semi-hard cheese inoculated with *E. coli* strains. After subjecting the model semi-hard cheese to pressure of 300 MPa for 10 minutes and at 500 MPa for 5 minutes, the counts of *E. coli* O157:H7 strain ATCC 43894 decreased by 1.3 log units and 3.7 log units, respectively, in comparison to the control cheese on day 2. On day 50, the log unit reduction increased to 3.8 and 5.8 after high pressure treatment at 300 MPa for 10 minutes and at 500 MPa for 5 minutes, respectively, with an initial temperature of 10 C. This research indicates that the application of pressure during cheese ripening is a feasible procedure to reduce present pathogens and improve cheese safety. Furthermore, cheese can be subsequently contaminated during its production. *Listeria monocytogenes* can be found on the equipment, such as vats, pipes, and other utensils, used to

make cheese that are not properly cleaned and sanitized. Cheese production environment is always moist and there are numerous locations where water and organic residues can be retained. The aforementioned environment favours the activity and development of *Listeria* that additionally has the ability to generate and form biofilm that serves as a further protection. In a research study done by Alves & Pérez (2020) mixed cheese was inoculated with twelve strains of *Listeria monocytogenes*, and then subjected to processing under 600 MPa pressure for one, three, five, and ten minutes. Inactivation levels ranged from 1.25 to 6.49 Log CFU/g. Based on the findings, the processing parameters most effective for eliminating *L. monocytogenes* in the product were identified as 600 MPa pressure for five minutes. Therefore, this research affirms the effectiveness of high pressure treatment on pasteurized cheese in decreasing the prevalence of *L. monocytogenes* contamination. Other than cheese production, ripening and storage conditions also contribute to the contamination hazards and spoilage. Cheese ripening chambers provide a unique environment that favours the growth of contaminants such as moulds, yeasts, and undesirable bacteria. Considerable efforts have therefore been directed toward optimizing the ripening process and improving the microbiological quality of ripened cheese. By reducing the concentration of undesirable microorganisms in cheese and inactivation of their enzymes, other unwanted formations such as biogenic amines can also be affected.

Biogenic amines are non-volatile nitrogen organic bases of low molar mass that are part of the metabolism of plants, animals and humans, and are naturally present in various types of food such as vegetables, fruit, meat and fish, while their presence in fermented products such as wine, beer, sausages and cheese is primarily a product of microbiological activity (Bäumlisberger *et al.*, 2015). Cheese contains two major biogenic amines, histamine and tyramine, which are mostly produced by the decarboxylase activity of *Enterococcus* spp. and *Lactobacillus* spp. strains. The precursors of the mentioned amines are free amino acids generated by the hydrolysis of milk proteins. During a 240-day study, Calzada *et al.* (2015a) evaluated cheeses made with unpasteurized milk were either treated with 400 or 600 MPa after ripening for 14 or 21 days and their characteristics were compared to untreated control cheese. Results revealed a decrease in counts of lactic acid bacteria and untraceable count of gram-negative bacteria, along with lower aminopeptidase activity in high pressure cheeses. Contrarily, levels of hydrophilic and hydrophobic peptides were higher than control cheese on day 60 and beyond. Total free amino acid concentrations were lower in 600 MPa cheeses after day 60. And finally, all high pressure treated cheeses had lower concentrations of total biogenic amines than the control cheese. In their study, Espinosa-Pesqueira *et al.* (2018) investigated the

impact of exposing artisanal Spanish cheeses to high hydrostatic pressure at 400 MPa for 10 minutes at a temperature of 2°C on the formation of biogenic amines (BAs). The high pressure treatment was administered both at the beginning of the ripening process and after 15 days. Results revealed that mentioned treatment had a significant effect on reducing the amount of BAs present in cheese after ripening. This reduction could be attributed to the substantial decrease in microbial counts, particularly in the lactic acid bacteria, enterococci, and enterobacteria species at the start of the process. Additionally, the proteolysis in samples was also altered, leading to a decrease in the quantity of free amino acids observed. Consequently, the application of positive pressure during ripening does not only cause proteolytic changes, it also induces other biochemical changes, which include lipolysis and glycolysis.

High pressure processing alters the microstructure and texture of cheese by affecting its main components - proteins and lipids. Cheese proteolysis can be affected by high pressure owing to the alteration in protein conformation, activation or inactivation of proteinases, and inhibition or enhancement of microbial growth and metabolism. Cheese subjected to high pressure contains lower levels of proteins, mainly caseins, and higher levels of hydrophilic peptides and hydrophobic peptides, which is to be expected due to induced protein hydrolysis. High pressure also affects enzymatic activity. The extent to which enzymes involved in cheese ripening are inactivated varies depending on factors such as enzyme type, substrate composition, and treatment conditions. Calzada *et al.* (2015a) reports decrease of bacterial peptidase and aminopeptidase activity during the ripening of high-pressured cheese. After the bacteria cell lysis, intracellular enzymes were released and exposed to the pressure treatment which caused their inactivation. Consequently, the level of free amino acids in the pressure-treated cheese did not vary much from the control cheese. However, naturally occurring milk protein, plasmin, is not degraded and retains its activity even after applying very high pressure in the cheese because of its baroresistance. High pressure may also have an impact on the formation of free fatty acids and volatile compounds in cheese. The same authors conducted different research on the influence of high pressure processing on the lipolysis, volatile compounds and sensory characteristics of unpasteurized milk and found out that high pressure levels didn’t have much of an effect on the quantity of free fatty acids in cheese but reported a higher levels of ketones, hydrocarbons, aldehydes and sulhur compounds (Calzada *et al.*, 2015b). It is evident that enzymatic changes under the influence of pressure affect the breakdown of proteins and lipids, ultimately shaping the formation of flavour compounds. Different pressure treatments and holding time causes different changes. For example,

researchers have determined that cheese treated with low pressure range (below 200 MPa) but for a longer time (3 – 4 days), causes changes in the formation of various aroma and flavour compounds as it effects primary proteolysis. On the other hand, high pressure (200 – 500 MPa) for a shorter holding times (20 – 30 minutes) improved the functional and rheological properties of cheese due to the changes in protein structure (O'Reilly *et al.*, 2003; Ozturk *et al.*, 2013). The results of higher proteolysis and lipolysis are suggesting that high pressure treatment can reduce the ripening time of cheese as the main purpose of cheese ripening is to develop and enhance its flavour, texture, and aroma. The possibility of accelerating the ripening process of Reggianito Argentino cheese was investigated by Costabel *et al.*, (2016) where they applied pressure to 1-day old cheese with 100 or 400 MPa for 5 or 10 min at 20°C and compared it to control cheese that was not pressurized. Higher pressure treatment cheese showed an increased breakdown of casein fractions α_{S1} and β causing an increase in soluble peptides and free amino acids. Content of peptides and amino acids in a 60-day old cheese were comparable to those found in fully matured control cheeses that usually ripens for 90 days. The researchers concluded that treatment of 400 MPa increased the rate of proteolysis leading to an acceleration of the ripening. Some researchers also demonstrated that the combination of both high pressure and elevated temperature increases the rate of cheese proteolysis. This is known as pressure-assisted thermal processing (PATP) or high-pressure high-temperature processing. The application of both high pressure and elevated temperature can lead to enhanced protein denaturation and unfolding, ultimately increasing the activity of proteolytic enzymes. However, it is important to carefully optimize and control the pressure and temperature conditions to avoid excessive protein denaturation or degradation. Despite the studies carried out, further research is needed to define the optimal effects of high-pressure treatments on cheese and achieve the right balance of positive effects.

Devices that apply high pressure to cheeses are typically constructed using durable and robust materials to withstand the immense forces involved. General outline of the construction includes a pressure vessel, pressure generation system, temperature control device and materials handling system (Hokmollahi & Ehsani, 2017). The pressure device must consist of a sturdy frame that is made of materials like stainless steel or carbon steel, that in the same time provides structural support and holds all the components together. Pressing plates that exert pressure on the cheese are large and flat-surfaced, also made of stainless steel or food-grade aluminium to ensure hygiene and resistance to corrosion. Furthermore, to avoid any leaks or seepage, the pressing plates are often designed with a sealing mechanism. This ensures that the pressure is

uniformly distributed across the surface of the cheese and minimizes any loss during the process. This evenly distributed pressure, also known as isostatic pressure, allows the cheese, which is subjected to the application of high pressure, not to be crushed during the treatment. The pressure mechanism in these devices includes hydraulic or pneumatic systems. Hydraulic systems are more common and involve a hydraulic pump, hoses, and cylinders. Pneumatic systems, using compressed air, are occasionally used but may not be as capable of achieving very high pressures compared to hydraulic systems. One of the main and most important parts of high-pressure device is the pressure control. Pressure control features like pressure gauges, valves, and regulators are used to monitor and control the applied force. These components allow operators to adjust and maintain the desired pressure level throughout the process. Some high-pressure cheese devices may include a control system with programmable features that enables to set specific pressure and duration parameters for consistent and repeatable results. When pressure is applied, the rise in temperature occurs, which is why a medium is required to control the temperature. Cooling or heating medium, usually air, is being pumped through the jacket of the pressure vessel for a constant temperature (Hokmollahi & Ehsani, 2017). And lastly, high-pressure cheese devices often incorporate safety features to prevent accidents or damages. These may include emergency stop buttons, pressure relief valves, and sensors to ensure safe operation. Cheese before subjected to high pressure is, usually, vacuum packaged to withstand the high-pressure processing while preserving its quality and safety. Overall, the construction of high-pressure cheese devices focuses on durability, precision, and safety to effectively apply and control the high pressures needed for cheese processing.

In general, the application of overpressure during ripening shows that it contributes to the positive characteristics of cheese such as longer shelf life, better retention of nutrients, acceptable sensory quality (e.g. reduction of bitterness, acceptable colour, lower risk of early or late bloating) and is generally considered safe. In the particular case of cheese, the stage of ripening at which high pressure is applied is also a crucial parameter for the retention of sensory characteristics. However, cheeses treated with overpressure show greater syneresis and enhanced primary and secondary proteolysis, but on the other hand, excessive pressure levels can inactivate enzymatic activities and slow down biochemical reactions involved in cheese ripening (Nájera *et al*., 2021). Additionally, excessive pressure can cause headaches for employees if it is applied in large-scale, and the cost of installing and maintaining high-pressure equipment is relatively high. This means that the process may not be suitable for small-scale

producers. Nevertheless, as the demand for natural and healthy food products increases, high pressure is becoming more affordable and accessible.

Although high-pressure processing has been widely employed in cheese production, including for the non-thermal pasteurization of milk, during curd formation, shortly after manufacture, and even following the ripening stage, as reported by Nunez *et al.* (2020), its application within a specially designed pressurized ripening chamber during the cheese maturation process has not yet been investigated. This gap constitutes the central subject of the present scientific study.

3. EXPERIMENTAL PART

3.1. Materials and Methods

3.1.1. Milk collection

Raw whole cow's milk was sourced from a local dairy farmer in Karlovac (Croatia) in 2023, stored overnight, and then transported cold (+4°C) in PVC buckets to the dairy practicum of Karlovac University of Applied Sciences, where the cheese production and ripening took place.

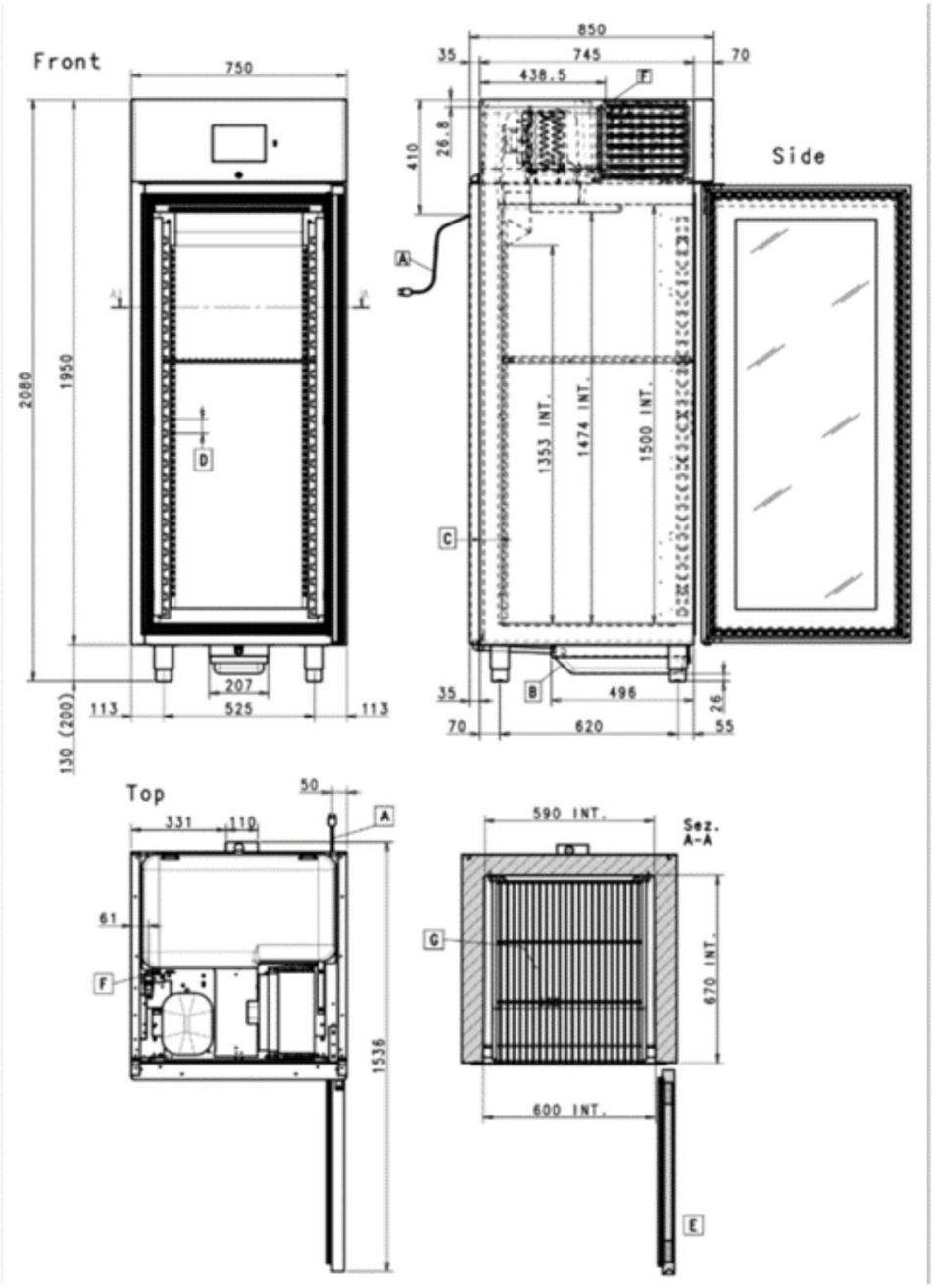
3.1.2. Cheese production

Upon milk reception, the production of semi-hard cheese was initiated by applying Low Temperature Long Time (LTLT) vat pasteurization, in which the milk was heated at 63–65°C for 25–30 min. The cheese was manufactured in a 30 L double-jacketed vat, using tap water as the heating and cooling medium. Thereafter, a lyophilized starter culture was added to the pasteurized milk at a concentration of 0.00182% (w/v) (0.546 g) (MYStarter CT 330 Series, *Streptococcus salivarius* ssp. *thermophilus*; Maysa Gida, Turkey), followed by the addition of microbial rennet at 0.00777% (w/v) (2.33 g) (MUCOREN 2000, *Rhizomucor miehei*; Maysa Gida, Turkey). After coagulation, the curd was cut, drained, moulded, and pressed under a pressure of 1–1.5 bar. The final step in cheese manufacture consisted of brining the cheese for 24 h in a brine solution containing 13% salt, with the pH adjusted to 4.5–4.7.

3.1.3. Cheese ripening

Ripening of cheese was carried out in two specially designed ripening chambers (Fasek d.o.o., Zagreb, Croatia), a control chamber and an overpressure chamber (**Figure 2**), with temperature, humidity, and airflow control option on a customizable local touch screen display.

The SCADA system was used to continuously monitor and regulate temperature (+0°C to +30°C), relative humidity (40% to 95%), airflow velocity (1 × Ø200 mm, 10 W), overpressure, and the concentrations of carbon dioxide and oxygen. In addition to process control, the system enabled trend recording, graphical and statistical data analysis, alarm notifications and email alerts, automated report generation in PDF and CSV formats, as well as remote monitoring and control. Both ripening chambers were fitted with audio-visual alarms for preset operating parameters, and alarm thresholds could be adjusted for all monitored variables.



A = Electrical connection L = 2100 mm
B = Condensate drainage tray
C = Insulation thickness 75 mm
D = Shelf spacing 53 mm
E = Right door opening / Left door opening – optional
F = Water connection ¾"
G = Stainless steel wire shelves 530x650 mm

Figure 2. Process scheme of ripening chambers

The internal dimensions of the ripening chamber were 530 × 650 × 1500 mm (W × D × H). Each chamber contained five mobile polypropylene shelves and had a product capacity of 100–150 kg. The control chamber (chamber 1) and the overpressure chamber (chamber 2) shared identical technical characteristics, except that chamber 2 was additionally equipped with a pressure regulation system enabling overpressure control within a range of 0.1–5.0 mbar.

Cheese samples were ripened under different pressure conditions, namely atmospheric pressure (1.01325 bar) in chamber 1 and elevated pressures ranging from 1.01443 to 1.01656 bar in chamber 2, for periods between 3.59 and 6.41 weeks, in accordance with the RSM and CCRD experimental design described in detail in the section *Experimental Design and Statistical Analysis*, as presented in **Table 2**. During ripening, the temperature was initially maintained at 10°C, increased to 16°C during the middle phase of the process, and subsequently reduced again towards the end of ripening. A similar pattern was applied to relative humidity, which began at 75%, reached a maximum of 85% in the middle of the ripening period, and then gradually returned to 75% by the final stage.

It should be emphasized that no routine cheese-care treatments were applied during ripening, such as brushing, washing, or coating with protective agents. The only intervention performed was the daily turning of the cheeses during the initial phase of ripening.

Table 2. The uncoded and coded levels of independent variables used in the Response surface methodology (RSM)

Independent variables	**Symbols**	**Levels**				
		-1. 414	**-1**	**0**	**+1**	**1. 414**
Overpressure (mbar)	X_1	1.18	1.50	2.25	3.00	3.31
Time (week)	X_2	3.59	4	5	6	6.41

3.1.4. Microbiological analyses

Raw milk, pasteurized milk and cheese were analysed for microbiological quality. Before the start of cheese production, 1 L of raw milk was excluded, and during production, 1 L of pasteurized milk and 1 L of whey. Different batches of cheese during ripening were also analysed after week 1, 2, 3, 4, 5 and 6, both in chamber 1 and 2.

3.1.5. Conducting microbiological analyses on cheese samples

Determining yeasts and moulds according to HRN ISO 21527-1:2012 (Horizontal method for the enumeration of yeasts and moulds - Part 1: Colony count technique in products with water activity greater than 0.95) and HRN ISO 21527-2:2012 (Horizontal method for the enumeration of yeasts and moulds - Part 2: Colony count technique in products with water activity less than or equal to 0.95). The methods include sample preparation, inoculation, and incubation of the test sample. Determining the presence of *Salmonella* spp. bacteria using the method HR EN ISO 6579-1:2017 (Horizontal method for the detection, enumeration, and serotyping of *Salmonella*). Determining the presence of *Listeria monocytogenes* bacteria using the method HR EN ISO 11290-1:2017 (Horizontal method for the detection and enumeration of *Listeria monocytogenes* and other *Listeria* spp.).

3.1.6. Experimental design and statistical analysis

Response surface methodology (RSM) and central composite rotatable design (CCRD) was used for determining optimal conditions (Bas & Boyaci, 2007) for cheese ripening using overpressure ripening chamber. The considered parameters during cheese ripening optimization were as follows: overpressure (1.18 – 3.31 mbar), and time (3.59 – 6.41 weeks) (Table 1). Experimental data were fitted with second order response surface model with the following form:

$$y = \beta_0 + \sum_{j=1}^{k} \beta_j X_j + \sum_{j=1}^{k} \beta_{jj} X_j^2 + \sum \sum_{i<j} \beta_{ij} X_i X_j \qquad (1)$$

where y is response (the main detected volatile compounds, respectively), β_0, β_j, β_{jj}, β_{ij} are constant coefficients of intercept, linear, quadratic, and interaction terms, respectively; Xì and Xj are coded independent variables [overpressure (X_1), and time (X_2)]. Ripening temperature and relative humidity were not included in the optimization process because they were constant for all types of cheese in both chambers. The response values were mean of the replicate measurement. Analysis was performed using commercial software Design-Expert® (ver. 12, Stat-Ease Inc., USA). The overall predictive capability of the model is commonly explained by the coefficient of determination (R^2). The analysis of variances (ANOVA) was also used to evaluate the quality of the fitted model. The test of statistical difference was based on the total error criteria with a confidence level of 95.0%. The lack-of-fit is significant at $p<0.05$ showing the adequacy of the quadratic model selected.

3.1.7. GC-MS analysis of volatile components

Gas chromatographic–mass spectrometric (GC–MS) analyses were carried out employing an Agilent Technologies gas chromatograph (model 7890A, Palo Alto, CA, USA) interfaced with a 5975C mass selective detector (Agilent Technologies). Separation was achieved on an HP-5MS capillary column (30 m × 0.25 mm i.d., 0.25 μm film thickness) coated with a 5% phenyl-methylpolysiloxane stationary phase. Helium was used as the carrier gas at a constant flow rate of 1 mL/min. The injector temperature was set to 250 °C. The oven temperature program consisted of an initial isothermal step at 70 °C for 2 min, followed by a linear ramp from 70 °C to 200 °C at 3 °C/min, and a final isothermal hold at 200 °C for 15 min. The injection was performed in split mode with a 1:20 split ratio. Electron impact ionization was applied at 70 eV, with an ion source temperature of 230 °C and a quadrupole temperature of 150 °C. Mass spectra were recorded within the scan range of 30–450 amu.

Relative percentages of volatile constituents were determined from the GC peak areas, calculated as mean values from duplicate GC–MS runs of each cheese sample.

For the solid-phase microextraction (SPME) procedure, a DVB/CAR/PDMS fiber (80 μm film thickness, Supelco Co., Bellefonte, PA, USA) was employed. Prior to use, the fiber was conditioned at 250 °C according to the manufacturer's instructions. Cheese samples (1 g) were transferred into 20 mL glass vials containing 5 mL of distilled water and 0.5 g NaCl, subsequently sealed with a PTFE/silicone septum. The PAL autosampler system was used to expose the SPME fibre to the headspace volatiles for 40 min under intermittent agitation. Following extraction, the fibre was thermally desorbed in the GC–MS injector (250 °C) for 7 min, after which the analytical run commenced.

Compound identification was accomplished by comparing the obtained mass spectra with reference spectra from the Wiley9 (New York, NY, USA), NIST11 (Gaithersburg, MD, USA), and Pest libraries integrated in the Agilent ChemStation software (OpenLab CDS 2.7, Agilent Technologies, Palo Alto, CA, USA).

3.1.8. Physicochemical analysis

The physicochemical composition of the cheese samples, fat content, moisture content, protein content, and salt content, was determined using a FoodScan™ Lab near-infrared analyser (FOSS, Hillerød, Denmark). The instrument operates on the principle of near-infrared transmittance (NIT) spectroscopy, employing polychromatic infrared radiation in the wavelength range of 850 – 1048 nm. As the incident light passes through the sample, the

principal cheese constituents, fat, water, and protein, absorb radiation at characteristic wavelengths; the resulting transmittance spectra are acquired and processed to yield quantitative compositional data. To ensure representative sampling, each cheese sample was rotated and scanned at 25 discrete positions during measurement, thereby accounting for potential heterogeneity within the cheese matrix. Spectral data were processed using pre-developed and independently validated global calibration models based on artificial neural networks (ANN), in accordance with the manufacturer's specifications and the requirements of ISO 21543/IDF 201:2006, which governs the application of infrared spectroscopy to the analysis of milk and dairy products. Each analysis was completed within approximately 45 to 60 seconds, without the use of chemical reagents. All measurements were performed in duplicate, and results are reported as mean values.

3.1.9. Sensory Analysis

All sensory tests were conducted in the Sensory Laboratory of the Karlovac University of Applied Sciences. Due to the same experimental design (overpressure and time), a total of 23 cheese samples were analysed, including 13 samples from the overpressure chamber and 10 samples from the control chamber. After each ripening treatment had been completed, sensory evaluation was carried out on all cheeses belonging to that specific treatment group, including one sample from the control chamber.

The trained descriptive panel consisted of five sensory assessors, employees of Karlovac University of Applied Sciences, both male and female, with an average age of 40 years. Before the formal evaluation, panel training and a group discussion were conducted in two 30-minute sessions using a reference cheese, during which a list of attributes was agreed upon, and reference intensities were calibrated. The sensory assessment was conducted between June and October 2023.

Prior to sensory evaluation, each cheese sample was equilibrated to a serving temperature of 12–14 °C, assigned a unique code, and presented to assessors within a single-blind testing design. The analysis was performed using Quantitative Descriptive Analysis (QDA), a descriptive sensory method. The sensory panel predefined a set of relevant sensory attributes for evaluation, including taste, aroma, texture, aftertaste, and crust/surface appearance **(Table 3)**. During assessment, the intensity of each attribute was quantitatively rated on a standardized scale, resulting in a detailed sensory profile for every cheese sample in accordance with the sensory profiling methodology described by ISO 13299:2016. The entire evaluation process

adhered to the relevant international standards (ISO 13299:2016, ISO 8586:2023, and ISO 8589:2007), ensuring objectivity, reliability, and reproducibility of the sensory results.

Table 3. Evaluation sheet for assessing the sensory properties of semi-hard cheese samples

DATE:				
EVALUATOR:				
EVALUATED ATTRIBUTE	*Please enter the achieved score for each attribute in the column of the corresponding sample.* *Evaluate the attributes in the order listed below.*			
	Sample 1	**Sample 2**	**Sample 3**	**Sample 4**
TASTE				
AROMA				
TEXTURE AND AFTERTASTE IN THE MOUTH				
APPEARANCE OF CRUST / SURFACE				

To obtain the evaluation score of the cheese samples, it is necessary to calculate the average score given by the panellists/tasters for each attribute and multiply it by the assigned weighting factor, as shown in **Table 4**. The resulting weighted scores are then summed for all evaluated attributes of the same cheese sample, and the final total must not exceed 20.

Table 4. Sensory Evaluation Criteria and Scoring System for Cheese Samples

Sensory attribute	**Descriptive parameters**	**Rating**	**Significance factor / Weighting factor**	**Max. points**
Taste	Clearly expressed, characteristic of the product, free of off-flavours, moderate aroma, moderately salty	4-5	2.0	10.0
	Overly pronounced milk flavour, weak aroma, insufficient saltiness, traces of acidity, bitterness or rancidity, rind flavour, traces of foreign flavours	3		

	Foreign flavour, uncharacteristic taste, rancid, sour, bitter, overly salty, completely unsalted (bland), excessively intense aroma, mouldy flavour	1-2		
Aroma	Pleasant, neither too strong nor too weak, characteristic milky aroma, mild acidic note, no foreign odours	4-5	1.5	7.5
	Overly intense or insufficient aroma, weak milk aroma, traces of rancidity	3		
	Completely uncharacteristic, foreign, rancid, mouldy aroma	1-2		
Texture and Aftertaste in the Mouth	Compact and homogeneous cheese, firmness characteristic of the product, smooth and even cut surface, uniform colour throughout, no lumps, does not stick to the mouth	5	0.3	1.5
	Noticeable minor irregularities and indentations, slightly too hard or too soft, minor inhomogeneities visible on the cut surface	3-4		
	Too hard or too soft, uneven granulation and colour, sandy or crumbly texture, noticeably sticks to the mouth	1-2		
Appearance of Rind / Surface	Homogeneous, smooth, glossy, uniform over the entire surface	5	0.2	1.0
	Uneven, slightly rough surface, noticeable colour non-uniformity on the rind	3-4		

	Cracked rind, completely uneven and rough surface, noticeable zones of different rind colours, foreign and uncharacteristic rind or surface colour	1-2		
TOTAL WEIGHTED SCORE:				**20**

4. RESULTS

The high-pressure processing is extensively used in the cheese production, as already stated, high-pressure technology has been mainly applied as a short-term treatment to improve cheese safety and shelf-life (e.g., via inactivation of pathogens and spoilage enzymes) but it has never been applied in specially designed ripening chamber during cheese maturation like in this study. So, it was interesting to compare data obtained with classic cheese ripening with cheese ripening in overpressure chamber in terms of volatile compounds and microbiological analysis.

4.1. Microbiological Analyses

Microbiological analyses of cheese samples showed the absence of pathogenic and unwanted microorganisms; the total number of microorganisms was within the limits. By reviewing in **Table 5** and **Table 6**, it can be seen that the number of yeasts and moulds in both the control and overpressure chamber were generally maintained at low levels (< 2.00 log CFU/g) throughout all ripening times. This indicates that the cheese ripening process under controlled conditions effectively managed yeast growth. However, mould levels displayed varying trends. Overpressure levels of 2.25 and 3 mbar led to an increase in mould numbers during the first three weeks of ripening. After week 3, the mould count began to decline until the end of the ripening period, suggesting that the initial phase of mould growth was followed by a reduction, possibly due to the extended pressure treatment or factors such as competition for nutrients, or changes in environmental conditions (e.g., pH, moisture content, oxygen levels) that were influenced by the pressure treatment. It is clear that the different pressure levels had an impact on the growth dynamics of yeasts and moulds.

Notably, the consistent absence of pathogens like *Salmonella* and *Listeria monocytogenes* throughout ripening underscores the efficacy of the hygienic and pressure-controlled conditions in ensuring safety. The low counts of yeasts suggest that the starter culture and ripening conditions suppressed excessive yeast proliferation, which is beneficial as wild yeasts can otherwise cause spoilage or off-flavours. The transient increase in mould counts under the higher pressure (2.25 and 3 mbar) followed by a decline indicates that while moulds can initially grow even in a pressurized environment, likely due to sufficient oxygen and nutrients early on, the sustained overpressure eventually creates conditions unfavourable for mould survival or sporulation. Elevated pressure could reduce gas exchange or gradually increase dissolved CO_2 levels, creating a more inhibitory environment for aerobic moulds. Additionally, nutrient competition and the accumulation of fermentation by-products over time may have curtailed mould growth. These findings illustrate how slight modifications in pressure can markedly

influence microbial succession during cheese ripening, ultimately contributing to both improved safety and the development of desired organoleptic properties.

Table 5. Microbiological quality of cheese samples obtained in control chamber compared to the cheese samples obtained in an overpressure chamber with a 2.25 mbar

Number of yeasts		
Ripening time (weeks)	**Control chamber (log CFU/g)**	**Overpressure (log CFU/g)**
1	<2.00	<2.00
2	<2.00	3.00
3	<2.00	2.48
4	>4.18	<2.00
5	4.08	<2.00
6	< LOD	< LOD
7	2.78	2.40
Number of moulds		
Ripening time (weeks)	**Control chamber (log CFU/g)**	**Overpressure (log CFU/g)**
1	<2.00	<2.00
2	3.46	3.38
3	>4.18	3.84
4	>4.18	2.65
5	3.45	2.18
6	< LOD	/
7	3.53	3.15
Number of *Escherichia coli B-Glucuronidase+*		
Ripening time (weeks)	**Control chamber (log CFU/g)**	**Overpressure (log CFU/g)**
1	< 1.00	< 1.00
2	< 1.00	< 1.00
3	< 1.00	< 1.00
4	2.52	< 1.00
5	< 1.00	< 1.00
6	< 1.00	< 1.00
7	< 1.00	< 1.00
Number of Coagulase positive *Staphylococcus 37°C*		
Ripening time (weeks)	**Control chamber (log CFU/g)**	**Overpressure (log CFU/g)**
1	< 0.00	< 0.00
2	< 0.00	< 0.00
3	< 0.00	< 0.00
4	< 0.00	< 0.00
5	< 0.00	< 0.00
6	< 0.00	< 0.00
7	< 0.00	< 0.00
Number of *Salmonella* spp.		
Ripening time (weeks)	**Control chamber (log CFU/g)**	**Overpressure (log CFU/g)**
1	< LOD	< LOD
2	< LOD	< LOD
3	< LOD	< LOD
4	< LOD	< LOD
5	< LOD	< LOD
6	< LOD	< LOD
7	< LOD	< LOD
Number of *Listeria monocytogenes*		

Ripening time (weeks)	Control chamber (log CFU/g)	Overpressure (log CFU/g)
1	< LOD	< LOD
2	< LOD	< LOD
3	< LOD	< LOD
4	< LOD	< LOD
5	< LOD	< LOD
6	< LOD	< LOD
7	< LOD	< LOD

*****LOD - Limit of Detection

Table 6. Microbiological quality of cheese samples obtained in control chamber compared to the cheese samples obtained in an overpressure chamber with a 3 mbar

Number of yeasts		
Ripening time (weeks)	**Control chamber (log CFU/g)**	**Overpressure (log CFU/g)**
1	<2.00	<2.00
2	/	< LOD
3	<2.00	<2.00
4	<2.00	<2.00
5	<2.00	<2.00
6	<2.00	<2.00
Number of moulds		
Ripening time (weeks)	**Control chamber (log CFU/g)**	**Overpressure (log CFU/g)**
1	>4.18	2.40
2	< LOD	< LOD
3	3.95	3.88
4	3.91	3.34
5	3.46	3.80
6	2.48	<2.00
Number of *Escherichia coli B-Glucuronidase+*		
Ripening time (weeks)	**Control chamber (log CFU/g)**	**Overpressure (log CFU/g)**
1	< 1.00	< 1.00
2	< 1.00	< 1.00
3	< 1.00	< 1.00
4	< 1.00	< 1.00
5	< 1.00	< 1.00
6	< 1.00	< 1.00
Number of Coagulase positive *Staphylococcus 37°C*		
Ripening time (weeks)	**Control chamber (log CFU/g)**	**Overpressure (log CFU/g)**
1	< 0.00	< 0.00
2	< 0.00	< 0.00
3	< 0.00	< 0.00
4	< 0.00	< 0.00
5	< 0.00	< 0.00
6	< 0.00	< 0.00
Number of *Salmonella* spp.		
Ripening time (weeks)	**Control chamber (log CFU/g)**	**Overpressure (log CFU/g)**
1	< LOD	< LOD
2	< LOD	< LOD
3	< LOD	< LOD
4	< LOD	< LOD
5	< LOD	< LOD

6	< LOD	< LOD
Number of *Listeria monocytogenes*		
Ripening time (weeks)	**Control chamber (log CFU/g)**	**Overpressure (log CFU/g)**
1	< LOD	< LOD
2	< LOD	< LOD
3	< LOD	< LOD
4	< LOD	< LOD
5	< LOD	< LOD
6	< LOD	< LOD

***LOD - Limit of Detection

4.2. GC-MS Analysis of Volatile Components

Additionally, to optimize the cheese ripening process in a newly designed overpressure chamber, Response Surface Methodology (RSM) was applied. First introduced by Box and Wilson (1951), RSM and mathematical modelling have since become essential tools for process optimization. RSM is a collection of statistical and mathematical techniques used to model polynomial equations and initial data, which help describe the behaviour of a dataset for statistical predictions. This method is particularly valuable in optimizing, designing, developing, and improving processes where multiple variables influence the outcomes. In this experiment, a RSM was implemented to efficiently explore the two-factor space with a manageable number of trialshrcak.srce.hr. By fitting a second-order (quadratic) polynomial model to the results, the RSM approach provided not only the optimal combination of ripening time and overpressure for desired aroma outcomes, but also revealed how each factor and their interaction influenced the generation of volatile compounds. This statistical design of experiments is especially powerful in food processing studies because it can capture non-linear effects and pinpoint conditions for maximal or minimal formation of specific flavour compounds, which would be difficult to identify through one-factor-at-a-time experiments. Using the RSM technique, not only were optimized process parameters obtained, but also insights into the probability of achieving a solution and the system's sensitivity to changes in specific conditions within the experimental range. One of the key advantages of this approach is the ability to interpret results through graphical representations (3D plots), which enhance the understanding of the effects of independent variables. In this study, the impact of varying overpressure levels and cheese ripening time on volatile compounds (responses) was explored.

In **Table 7** is the screening of the obtained volatile compounds of cheese obtained in overpressure chamber compared to control sample.

Table 7. Screening of the obtained volatile compounds of cheese obtained in overpressure chamber compared to control sample (RI from the literature used for identifying the compounds).

			Area percentages (%)	
No	**Compound**	**RI**	**Control**	**Overpressure**
1	1-(3-methoxyphenyl) propan-2-amine	<800	0.2194	< LOD
2	4-(2-amino-1-hydroxypropyl) phenol	<800	0.0429	< LOD
3	Ala-gly, trimethylsilyl ester	<800	0.1332	0.1504
4	Ala-beta-Ala, trimethylsilyl ester	<800	0.2648	< LOD
5	2-Methylaminomethyl-1,3-dioxolane	<800	0.2597	< LOD
6	Benzeneethanamine, 2,5-difluoro-beta.,3,4-trihydroxy-N-methyl-	<800	0.2062	< LOD
8	Carbonic acid, ethyl-, methyl ester	<800	0.1393	< LOD
9	Methyl-2,3,5-tri-*O*-methyl-4-thio alpha d-arabinofuranoside	<800	0.051	< LOD
10	Carbonic acid, ethyl-, methyl ester	<800	0.0677	< LOD
11	2-Isopropoxyethylamine (2-(propan-2-yloxy) ethan-1-amine)	<800	0.0766	< LOD
13	dimethylsilanediol (dihydroxy(dimethyl)silane, dimethylsilanediol)	<800	0.0251	0.1004
14	2-Formylhistamine	989	0.0222	< LOD
16	Methylsylane	991	0.0102	< LOD
17	Hexanoic acid	991	7.56	2.4037
18	2-Nonanone (nonan-2-on)	1341	2.2251	< LOD
19	Benzoic acid	1345	1.8394	2.6898
20	Octanoic acid	1478	9.7839	3.2648
21	Benzothiazole	1592	0.5285	< LOD
22	(*E*)-2-Decenal	1604	0.472	< LOD
23	Tetrahydro-6-propyl-2*H*-Pyran-2-one	1649	0.5827	0.6147
24	2-Undecanone	1702	0.6113	0.3616
25	7-chloro-5-phenyl-1-trimethylsilyl-1,3-dihydro-2*H*-1,4-benzodiazepin-2-one	1734	0.3554	< LOD
26	2-Mercapto-5-methoxybenzoic acid	1747	0.6441	< LOD
27	Ethyl (*4Z*)-dec-4-enoate)	1795	0.1123	< LOD
28	n-Decanoic acid	1806	23.8087	9.2089

29	1,3-Cyclohexanediol (cyclohexane-1,3-diol)	1832	< LOD	0.1497
30	Ethyl octanoate	1841	0.1513	< LOD
31	Tetradecane	1847	0.2763	0.2856
32	Dimethyl phthalate (dimethyl benzene-1,2-dicarboxylate)	1857	1.8844	3.8387
33	Cyclopentanone, oxime (*N*-cyclopentylidenehydroxylamine)	1859	0.1717	< LOD
34	2H-Pyran-2-one, tetrahydro-6-pentyl- (6-pentyloxan-2-one)	1897	12.3708	13.6195
35	1,1,1,5,5,5-hexamethyl-3,3-bis[(trimethylsilyl)oxy] trisiloxane	1917	1.0182	< LOD
36	Dodecanoic acid	1922	7.17	4.0799
37	(S) (+)-Z-13-Methyl-11-pentadecen-1-ol acetate	1924	0.7103	< LOD
38	Dodecanoic acid	1959	0.3555	< LOD
39	Ethyl heptanoate	1963	0.2383	< LOD
40	Hexadecane	1965	0.9284	2.5618
41	1-Methylpyrrolidin-2-one	1972	0.4263	< LOD
42	Pentadecanal	1974	0.2589	1.2627
43	1,2,3,4,4a,5,8,9,12,12a-decahydro-1,4-methanobenzocyclodecene	1977	0.1286	< LOD
44	3,3'-Bicyclopentenyl	1980	0.5143	< LOD
45	2(*3H*)-Furanone, dihydro-5-(2- ctenyl)-, (Z)-(Dihydro-5-(2-octenyl)-2(3H)-furanone)	1986	0.4249	0.6194
46	3-Amino-2-phenazinol ditms (*N*-trimethylsilyl-3-trimethylsilyloxyphenazin-2-amine)	1990	0.3326	< LOD
47	Heptadecanal	2000	0.2168	< LOD
48	2(3H)-Furanone, 5-heptyldihydro- (5-heptyloxolan-2-one)	2003	2.014	2.5011
49	2-Tetradecanone	2007	0.7926	< LOD
50	2H-Pyran-2-one, 6-heptyltetrahydro- (6-heptyloxan-2-one)	2010	10.1006	< LOD
51	Tetradecanoic acid	2016	1.5314	1.9531
52	Oxacyclotetradecan-2-one, 13-methyl- (13-methyl-1-oxacyclotetradecan-2-one)	2022	0.0898	< LOD
53	Cyclooctane, (1,2-dimethylcyclooctane)	2040	0.8927	< LOD
54	Octadecanoic acid, 17-methyl-, methyl ester (Methyl 17-methyloctadecanoate)	2043	0.2545	< LOD
55	Heptadecane, (2,6,10,15-tetramethylheptadecane)	2050	0.6048	< LOD
56	Eicosane	2052	0.2715	0.2953
57	Oxirane, tetradecyl- (2-Tetradecyloxirane)	2053	0.688	0.4843

58	Mercaptosuccinic acid, tris(trimethylsilyl) ester (bis(trimethylsilyl) 2-[(trimethylsilyl)sulfanyl]butanedioate)	2057	0.2402	< LOD
59	Benzoic acid, 4-methyl-, 1-methylpropyl ester (1-methylpropyl 4-methylbenzoate)	2060	0.4083	< LOD
60	2-Hexadecene, 3,7,11,15-tetramethyl-, [R-[R*R*-(E)]]- ((2E,7R,11R)-3,7,11,15-tetramethylhexadec-2-ene)	2063	0.7013	0.9021
61	Benzene, (1-methyldodecyl)- (2-Phenyltridecane)	2067	0.1829	< LOD
62	2H-Pyran-2-one, tetrahydro-6-octyl- (6-Octyloxan-2-one)	2070	1.8392	13.0818 3.2741
63	n-Hexadecanoic acid	2092	0.5499	2.888
64	Trisiloxane, 1,1,1,5,5,5-hexamethyl-3,3-bis[(trimethylsilyl)oxy]- (1,1,1,5,5,5-hexamethyl-3,3-bis[(trimethylsilyl)oxy] trisiloxane)	2098	0.0684	< LOD
65	Pentasiloxane, dodecamethyl- (Dodecamethylpentasiloxane)	2105	0.4061	6.91
66	Ginsenol	2110	0.1982	< LOD
67	1-Octadecene	2142	0.1754	< LOD
68	Formic acid, 1-(4,7-dihydro-2-methyl-7-oxopyrazolo[1,5-a] pyrimidin-5-yl)-, methyl ester (Methyl 2-methyl-7-oxo-4,7-dihydropyrazolo [1, 5-a] pyrimidine-5-carboxylate)	2146	0.1637	< LOD
69	2-Amino-1-(o-methoxyphenyl) propane (1-(2-methoxyphenyl) propan-2-amine.)	2150	< LOD	0.0842
70	Benzenemethanol, 2-(2-aminopropoxy)-3-methyl- ([2-(2-aminopropoxy)-3-methylphenyl] methanol)	2159	< LOD	0.0674

LOD - Limit of Detection

According to that data it can be seen influence of selected overpressure process variables on the most abundant compounds detected using GC/MS (**Table 8**). The comparison of volatile profiles clearly shows that certain compounds were uniquely present or more pronounced in the overpressure-ripened cheese, whereas others dominated in the control sample. Even a moderate overpressure environment appears to shift metabolic pathways during ripening, suppressing some volatiles while enhancing the formation of others. This comprehensive screening provided the basis for focusing on the key aroma-active molecules in order to quantify how controlled pressure and ripening time influence these major aroma constituents.

Table 8. The experimental design and data for the response surface analysis (in %-percentage)

Run	Overpressure (mbar)	Time (week)	Hexanoic acid	Octanoic acid	n-Decanoic acid	3,5-dihydroxydecanoic acid 1,5 lactone	Dodecanoic acid	Delta-Dodecalactone
1	2.25	6.41	7.47	13.39	7.69	5.77	2.68	1.34
2	2.25	5	2.4	4.81	12.26	12.91	5.87	13.97
3	2.25	5	1.56	7.43	7.81	8.17	4.9	9.06
4	2.25	5	3.65	13.64	14.76	13.62	5.36	13.91
5	3.31	5	4.5	14.8	16.2	15.7	10.2	17.44
6	1.5	4	2.1	4.1	4.4	4.6	3.3	5.31
7	3	6	7.9	8.1	6.9	7.2	5.6	8.4
8	2.25	5	2.64	10.49	13.26	15.61	8.36	13.95
9	2.25	3.59	11.21	25.99	14.03	8.32	1.27	1.85
10	1.5	6	2.5	4.12	4.3	5.1	4.5	5.2
11	1.18	5	2.4	3.8	5.1	6.4	5.11	7.7
12	2.25	5	1.37	8.12	13.39	17.65	4.73	15.21
13	3	4	9.1	22.1	18.4	20.3	13.3	18.1

From the data obtained in **Table 8** it can be seen that the concentration of hexanoic acid is relatively low compared to other developed compounds ranging from 1.56 to 11.21%. This relatively low proportion of hexanoic acid suggests that its formation was limited under the applied conditions, which can be favourable since excessive hexanoic acid could dominate the cheese aroma with an overly pungent, goat-like note. Hexanoic acid, commonly referred to as caproic acid, is a saturated fatty acid naturally present in a variety of food products and characterized by a sharp, acidic, and goat-like odour. When present in moderate concentrations, it may positively contribute to the aroma profile of certain cheese varieties by imparting sweaty and cheesy sensory notes (Fan & Qian, 2006). Similarly, octanoic acid, or caprylic acid, also plays a role in the development of cheese aroma. This saturated fatty acid is associated with slightly sweet and fruity notes, but also with rancid, musty, waxy, and goat-like characteristics, and is commonly encountered in coconut oil as well as in selected dairy products (Altinoz et al., 2020). The highest levels of caprylic acid were observed in the cheeses from experimental

runs 9 and 13, which were ripened under pressures of 2.25 and 3 mbar for 3.59 and 4 weeks, respectively. Across all analysed samples, the concentration of octanoic acid exhibited considerable variation, spanning from 3.80% to 25.99%. Beyond its contribution to cheese flavour, caprylic acid has also been widely recognized for its antimicrobial properties (Nair et al., 2005).

This large variation in octanoic acid content among samples underscores how sensitive the formation of this compound is to the ripening conditions. In particular, the fact that moderate pressure combined with shorter ripening time (as in runs 9 and 13) yielded the highest octanoic acid levels implies a synergistic effect: under these specific conditions, microbial lipase activity or other biochemical processes might be especially effective at releasing medium-chain fatty acids. Notably, the RSM analysis showed a significant interaction between overpressure and time for octanoic acid production, confirming that both factors together influenced its accumulation.

Another saturated fatty acid with slightly rancid odour is n-decanoic (capric) acid. Capric acid is typically not a desirable component of cheese aroma. Its sour, rancid, and animal-flavour attributes are generally considered unpleasant and should be minimized during cheese production (Güler, 2005). The lowest concentrations were observed under the lowest overpressure treatment 1.19 and 1.50 mbar. This suggests that higher overpressure and/or longer ripening times tend to promote the formation of capric acid, since only the mildest pressure conditions kept its level minimal. Correspondingly, the statistical model confirms that increasing either factor significantly elevates n-decanoic acid concentrations (with both pressure and time terms showing $p < 0.05$. Caproic, caprylic, and capric acids have been reported in a range of cheese varieties, including aged Cheddar, Grana Padano, and especially goat cheese, where they may be considered desirable due to their contribution to the characteristic tangy or "goaty" aroma **(**Tian et al., 2020**)**. In the present study, the highest concentrations of these compounds were observed under the shortest ripening period and at an overpressure of 2.25 mbar. Nevertheless, when present in excessive amounts, these fatty acids may generate undesirable odour notes, indicating that their levels should be carefully monitored and controlled. During cheese manufacture, particularly in the initial stages of fermentation, triglycerides are degraded through the metabolic activity of lactic acid bacteria (LAB) and other microorganisms naturally present in milk, resulting in the release of several fatty acids, including hexanoic, octanoic, and decanoic acids. Previous studies have shown that the occurrence of 3,5-dihydroxydecanoic acid 1,5-lactone may be associated with the metabolic

activity of different fungal species **(**Laili et al., 2017; Mentle, 1987; Vesonder et al., 1972**)**. In the analysed cheese samples, this cyclic ester was identified as the most abundant aroma compound.

The predominance of this particular lactone suggests that the special ripening conditions might have enhanced the pathways of certain microorganisms (such as moulds or yeasts) that produce lactones, thereby strongly shaping the cheese's aroma profile. Lactones are known for imparting a rich and fatty character to the aroma profile of cheeses which would make them a desirable compound in cheese, as it can contribute to a creamy and slightly sweet aroma. Furthermore, dodecanoic acid, or lauric acid, is a saturated fatty acid with a twelve-carbon chain. It has soapy and waxy notes that can lead to off-flavours, and is not typically a desirable aroma compound in cheese and should be kept at low levels (Güler, 2005). Consequently, lower concentrations of lauric acid (1.27 – 13.30%) were found in cheese samples compared to other aroma components. In cheese, lauric acid at even modest levels is known to introduce perceivable soapy or waxy off-flavours, so the relatively low amounts observed here are favourable for the overall flavour quality. Delta-dodecalactone can be a desirable component in cheese aroma, as it imparts sweet and creamy notes. It is often associated with a pleasant buttery, caramel-like aroma, and its presence can enhance the sensory experience of certain cheese varieties. Higher concentrations were found at higher overpressure treatments (2.25 – 3.31 mbar). Indeed, delta-dodecalactone is reported to have a powerful creamy, fruity (peach/apricot), and coconut-like aroma, so its increase under the higher-pressure conditions likely enriched the cheese's buttery-sweet aromatic profile. Cheese samples with the same overpressure and time may have significantly different aroma components concentrations. It seems that neither overpressure (measured in mbar) nor time duration (in weeks) alone can fully explain the variation in aroma components concentration. This variability highlights the complex nature of flavour development, where even under identical nominal conditions, slight differences in microbial activity or micro-environment can lead to divergent volatile outcomes. It underscores the need for a multifactorial analytical approach (such as RSM) to account for these variations and suggests that additional factors or interactions (beyond just pressure and time) might influence the production of aroma compounds.

The effects of the linear, quadratic, and interaction coefficients on the response were assessed for statistical significance through analysis of variance (ANOVA). Regression coefficients corresponding to the intercept as well as to the linear, quadratic, and interaction terms of the model were estimated using the least squares approach. The statistical relevance of each factor

is indicated by its associated p-value. **Table 9** summarizes the p-values obtained for the selected response variables in relation to each coefficient and interaction term. From **Table 9**, it can be observed that overpressure had the most statistically significant effect ($p < 0.05$) on all six investigated responses. However, time had a statistically significant influence only on the levels of octanoic acid and n-decanoic acid. The interaction between overpressure and time significantly affected only the levels of octanoic acid and n-decanoic acid. This indicates that chamber pressure was the dominant factor in determining the concentrations of volatile compounds, whereas extending the ripening duration alone (within the studied range) did not significantly change most aroma levels unless pressure conditions were also altered. Octanoic acid and n-decanoic acid were unique in that they continued to respond to longer ripening and showed pressure–time interaction effects, suggesting that the formation or retention of these particular compounds depends on a combination of aging duration and pressure (perhaps reflecting ongoing microbial transformations or volatiles being produced and then consumed at different stages).

Table 9. Regression coefficient of polynomial function of all response surfaces

Term	Coefficients	Standard error	*F*-value	*p*-value*
Hexanoic acid				
Intercept	2.32	0.7363		
X_1	1.92	0.5821	10.89	0.0131
X_2	-0.7611	0.5821	1.71	0.2323
X_1X_2	-0.4000	0.8232	0.2361	0.6419
X_1^2	0.3142	0.6242	0.2535	0.6301
X_2^2	3.26	0.6242	27.26	0.0012
Octanoic acid				
Intercept	8.90	1.68		
X_1	4.69	1.33	12.41	0.0123
X_2	-3.97	1.33	8.91	0.0097
X_1X_2	-3.51	1.88	3.46	0.0204
X_1^2	-1.02	1.43	0.5116	0.1051
X_2^2	4.17	1.43	8.54	0.4976
n-decanoic acid				
Intercept	12.30	1.06		
X_1	4.04	0.8395	23.13	0.0019
X_2	-2.57	0.8395	9.38	0.0183
X_1X_2	-2.85	1.19	5.76	0.0474
X_1^2	-1.39	0.9002	2.37	0.1673
X_2^2	-1.28	0.9002	2.03	0.1975
3,5-dihydroxydecanoic acid 1,5 lactone				
Intercept	13.59	1.35		
X_1	3.87	1.06	13.23	0.0083
X_2	-2.03	1.06	3.63	0.0985
X_1X_2	-3.40	1.50	5.11	0.0583
X_1^2	-1.21	1.14	1.12	0.3247

X_2^2	-3.21	1.14	7.92	0.0260
Dodecanoic acid				
Intercept	5.84	0.8708		
X_1	2.29	0.6884	11.04	0.0127
X_2	-0.5632	0.6884	0.6694	0.4402
X_1X_2	-2.22	0.9735	5.22	0.0562
X_1^2	1.37	0.7382	3.45	0.1058
X_2^2	-1.47	0.7382	3.96	0.0868
Delta-dodecalactone				
Intercept	13.22	1.11		
X_1	3.72	0.8783	17.95	0.0039
X_2	-1.32	0.8783	2.25	0.1776
X_1X_2	-2.40	1.24	3.73	0.0949
X_1^2	0.2175	0.9418	0.0533	0.8240
X_2^2	-5.27	0.9418	31.31	0.0008
X_1:overpressure; X_2: time; * $p < 0.01$ highly significant; $0.01 \leq p < 0.05$ significant; $p \geq 0.05$ not significant.				

Analysis of variance (**Table 10**) shows that the regression models for all investigated responses were statistically relevant with a significance level ranging from $p <= 0.0031$ to $p = 0.0275$, and the models had no significant lack of fit ($p > 0.05$). The fitted model represents the experimental data well with high correlation coefficients, R^2, varying from 0.7843 to 0.8892, depending on investigated responses. The fairly high R^2 values demonstrate that the polynomial models explained a large portion of the variability in each response. Moreover, the absence of a significant lack-of-fit means the chosen model form was adequate for describing the data, with no indication of unmodeled systematic trends. This gives confidence that the RSM-derived insights and optimization are reliable and reflective of real process behaviour within the tested domain.

Table 10. Analysis of variance (ANOVA) of the modelled responses

Source	Sum of squares	Degree of freedom	Mean square	F-value	*p*-value
Hexanoic acid					
The recovery					
Model	108.79	5	21.76	8.03	0.0082
Residual	18.97	7	2.71		
Lack of fit	15.62	3	5.21	6.20	0.0551
Pure error	3.36	4	0.8394		
Total	127.76	12			
R^2 = 0.8515					
Octanoic acid					
The recovery					
Model	490.18	5	98.04	6.91	0.0123
Residual	99.32	7	14.19		
Lack of fit	54.82	3	18.27	1.64	0.3143
Pure error	44.49	4	11.12		
Total	589.50	12			
R^2 = 0.8315					
n-decanoic acid					
The recovery					
Model	237.71	5	47.54	8.43	0.0071
Residual	39.46	7	5.64		
Lack of fit	11.14	3	3.71	0.5244	0.6886
Pure error	28.32	4	7.08		
Total	277.17	12			
R^2 = 0.8576					
3,5-dihydroxydecanoic acid 1,5 lactone					
The recovery					
Model	274.93	5	54.99	6.08	0.0174
Residual	63.34	7	9.05		
Lack of fit	12.94	3	4.31	0.3423	0.7975
Pure error	50.40	4	12.60		
Total	338.27	12			
R^2 = 0.8127					
Dodecanoic acid					
The recovery					
Model	96.49	5	19.30	5.09	0.0275
Residual	26.54	7	3.79		
Lack of fit	17.84	3	5.95	2.74	0.1778
Pure error	8.70	4	2.17		
Total	123.02	12			
R^2 = 0.7843					
Delta-dodecalactone					
The recovery					
Model	346.59	5	69.32	11.23	0.0031
Residual	43.19	7	6.17		
Lack of fit	20.36	3	6.79	1.19	0.4199
Pure error	22.84	4	5.71		
Total	389.79	12			
R^2 = 0.8892					

The three-dimensional plots used to represent the investigated responses (y) as a function of independent variables (in terms of coded values) are presented in Figure 3. From Figure 3, it can be observed that as overpressure increases, the levels of all six responses (hexanoic acid, 3,5-dihydroxydecanoic acid 1,5-lactone, dodecanoic acid, delta-dodecalactone, octanoic acid, and n-decanoic acid) significantly rise. The effect of cheese ripening time varies across the observed responses. In general, the formation of aroma components increases with ripening time. The concentrations of hexanoic acid and octanoic acid show a slight decrease after the fourth week of ripening, but then increase as time progresses. In contrast, the concentrations of dodecanoic acid, delta-dodecalactone, and n-decanoic acid follow a positive trend. These aroma components increase throughout the ripening process, peaking around 5.5 weeks, before slightly decreasing towards the sixth week. These non-linear trends suggest that there are optimal windows during ripening for maximizing certain flavour compounds: beyond roughly 5–6 weeks, the rates of formation and degradation of some volatiles may reach equilibrium or even favour degradation/volatilization, resulting in a decline. For example, hexanoic and octanoic acids may initially accumulate but then get partly metabolized by microorganisms or evaporate from the cheese matrix, explaining their dip mid-process. Meanwhile, compounds like delta-dodecalactone and n-decanoic acid, which steadily increase for longer, might be tied to slower, continuous biochemical reactions or microbial processes that peak later in ripening.

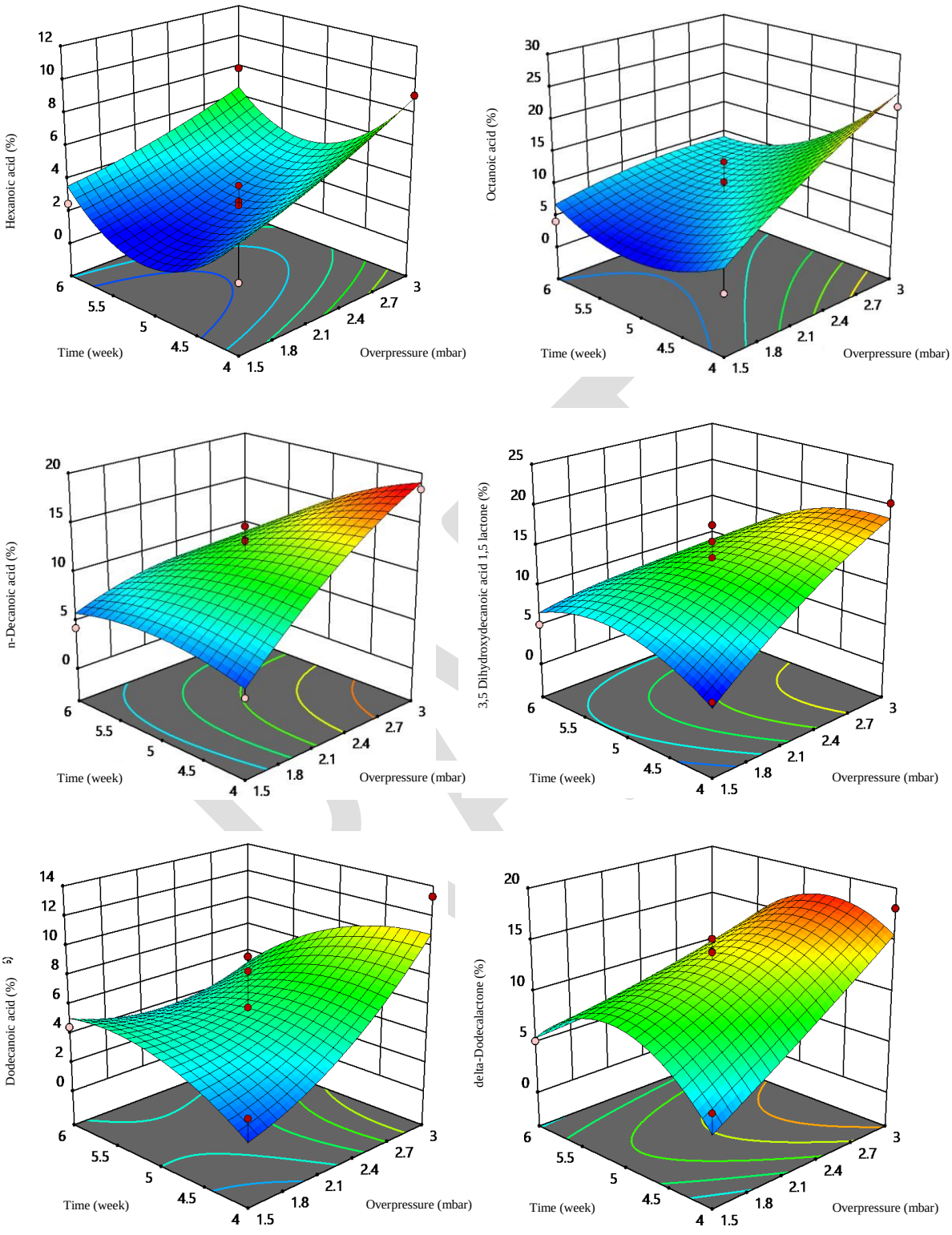


Figure 3. Three-dimensional plots for obtained responses as a function of time of cheese ripening and overpressure

4.3. Physicochemical Analysis

In addition to the microbiological analyses and GC-MS volatile compound profiling described in the preceding sections, the physicochemical composition of the cheese was determined for each of the 13 experimental runs at the end of the ripening period in order to provide a complete characterisation of product quality. The results for all 13 runs, together with the corresponding overpressure and ripening time conditions, are presented in Table 11.

Table 11. Physicochemical composition of semi-hard cheese ripened under different overpressure conditions

	Physicochemical composition			
Run	Fat (%)	Moisture (%)	Protein (%)	Salt (%)
1	33.27	29.48	28.59	1.46
2	33.13	32.99	26.59	1.78
3	32.59	32.38	27.01	1.71
4	32.39	33.55	26.27	1.98
5	33.01	35.77	24.90	2.34
6	32.67	33.58	26.48	1.88
7	32.79	33.15	26.69	1.85
8	32.30	34.17	26.80	1.81
9	31.29	37.65	25.05	2.25
10	32.98	32.28	27.04	1.75
11	34.27	30.18	27.16	1.67
12	32.33	34.11	26.88	1.78
13	32.47	34.45	26.13	1.98

Across all 13 experimental runs, fat content ranged from 31.29 % (Run 9; 2.25 mbar, 3.59 weeks) to 34.27 % (Run 11; 1.18 mbar, 5 weeks). Fat content showed an inverse relationship with moisture content across the experimental runs, consistent with the well-established concentration effect: as moisture is lost from the cheese matrix, the relative proportions of the remaining constituents, primarily fat and protein, increase accordingly. This pattern was most evident when comparing Run 9 (moisture 37.65 %, fat 31.29 %) with Run 1 (moisture 29.48 %, fat 33.27 %): the run with the lowest moisture content exhibited the highest fat content among the centre-point runs. Conversely, Run 11, conducted at the lowest overpressure (1.18 mbar), yielded both a low moisture content (30.18 %) and the highest fat content recorded across all runs (34.27 %), suggesting that at minimal overpressure the dominant process is conventional moisture evaporation rather than pressure-mediated moisture retention. The overall variability in fat content (31.29 – 34.27 %), and no sample approached the threshold of fat loss that would compromise the sensory or nutritional properties of the product.

Moisture content emerged as the parameter most strongly influenced by both overpressure and ripening time. The most pronounced moisture reduction was observed in Run 1 (2.25 mbar, 6.41 weeks; 29.48 %), compared to Run 9 (2.25 mbar, 3.59 weeks; 37.65 %), both conducted at identical overpressure but differing in ripening duration by approximately 2.8 weeks. This difference of 8.17 percentage points represents a substantial effect of ripening time on moisture retention and is consistent with the progressive evaporation of water from the cheese surface during extended maturation. A similar trend was observed when comparing runs conducted at equivalent ripening times but different overpressure levels. At approximately 5 weeks of ripening, Run 11 (1.18 mbar) showed a moisture content of 30.18 %, while Run 5 (3.31 mbar) reached 35.77 %, indicating that higher overpressure was associated with increased moisture retention under these conditions.

Protein content was inversely related to moisture content, as expected from the concentration mechanism. The highest protein values were recorded in runs with the lowest moisture: Run 1 (28.59 %, moisture 29.48 %) and Run 11 (27.16 %, moisture 30.18 %). The lowest protein content was found in Run 5 (24.90 %), which also showed one of the highest moisture contents (35.77 %) among the runs conducted at equal ripening time.

Salt content ranged from 1.46 % (Run 1) to 2.34 % (Run 5) and showed a pattern that is most directly explained by the variation in moisture content. Run 5, which combined the highest overpressure (3.31 mbar) with a moderate ripening time (5 weeks) and the highest moisture content (35.77 %), yielded the highest salt content (2.34 %), suggesting that the elevated moisture retention under maximum overpressure also enhanced salt uptake from the cheese surface brine, or that the moisture-rich matrix dissolved and distributed salt more effectively. Conversely, Run 1, the driest cheese (moisture 29.48 %), showed the lowest salt content (1.46 %), consistent with a more concentrated but less permeable matrix offering reduced salt diffusion pathways over the extended ripening period.

4.4. Sensory Analysis

The sensory analysis of cheese samples was conducted to evaluate the influence of increased overpressure during ripening on key sensory attributes, including aroma, taste, texture and aftertaste, as well as rind appearance. The evaluation was performed according to a structured sensory scoring system with a maximum of 20 weighted points. In total, 115 individual sensory evaluations were collected, ensuring sufficient data robustness and statistical relevance.

The results revealed clear differences among the 23 cheese samples in terms of aroma intensity, flavour complexity, texture, and overall sensory acceptability. Cheeses matured under overpressure conditions achieved higher average total sensory scores, ranging from approximately 16.8 to 18.3 points, compared to control samples from the standard chamber, which generally scored between 13.1 and 15.6 points.

A group of cheeses ripened under identical overpressure conditions and removed from the chamber at the same time (Runs 2, 3, 4, and 12) showed closely aligned overall sensory scores, ranging from 17.4 to 18.3 points. This relatively narrow distribution indicates good process repeatability and confirms that the applied ripening conditions resulted in consistent sensory outcomes. The slightly higher score observed for Run 12 suggests a marginally more advanced flavour development, while still remaining within the expected variability for cheeses produced and ripened under identical conditions.

Among all overpressure-treated samples, Run 12 achieved the highest overall sensory score (18.3 points), indicating the most favourable balance of aroma intensity, flavour complexity, texture, and aftertaste. Runs 9 and 13 also exhibited high sensory acceptability, with mean scores close to 18 points, while Run 11 achieved a slightly lower but still elevated score (17.7 points), confirming good but not exceptional sensory performance.

The remaining overpressure-treated samples exhibited moderate to high sensory scores (approximately 16.8 - 17.6 points), reflecting generally good sensory quality, although with slightly reduced aromatic intensity or flavour persistence compared to the highest-ranked runs.

In contrast, several control samples, particularly those from early production runs with shorter ripening times, received lower sensory scores, especially in aroma and taste. These cheeses typically achieved total scores between 13 and 14 points and were described as “mild,” “underdeveloped,” or “flat.” One of the lowest-rated samples originated from the control group ripened for only 3.59 weeks (Run 9), with an average total score of 13.8 points. The distribution of individual sensory scores assigned by the evaluators (1 to 5) for cheese samples (control and overpressure) is presented in **Figures 4 – 16**.

Overall, the sensory evaluation results based on 115 assessments clearly indicate that overpressure-assisted ripening enhances sensory quality while also demonstrating good reproducibility of sensory outcomes when cheeses are ripened under identical technological conditions.

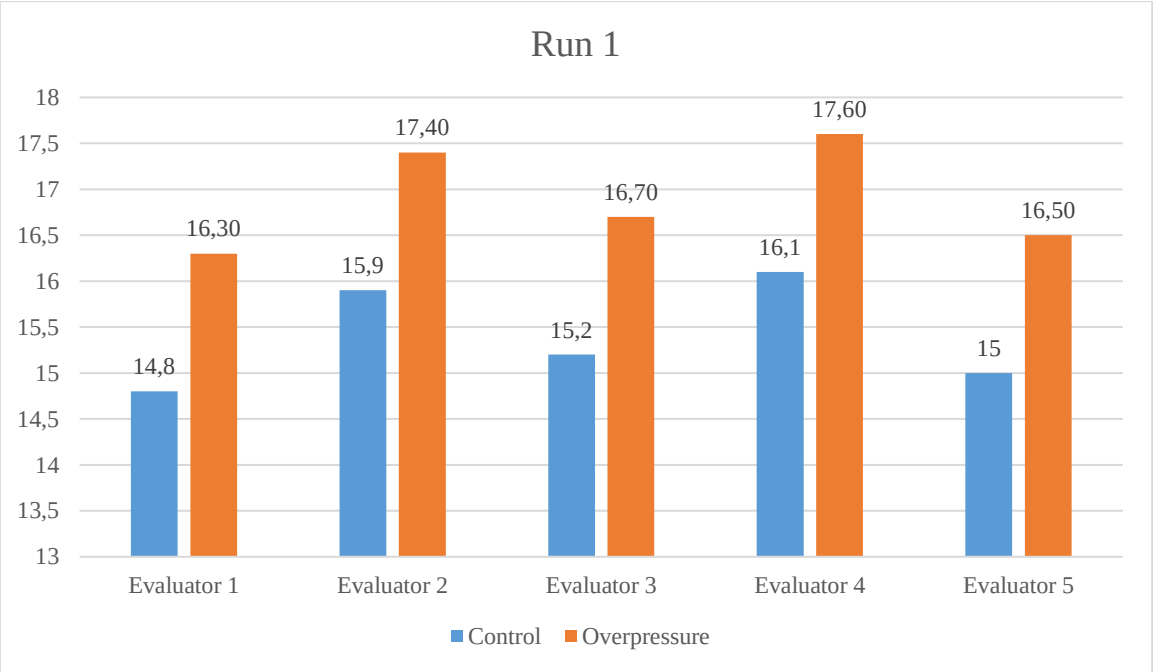


Figure 4. Run 1 – Individual sensory scores of cheese samples (overpressure vs. control)

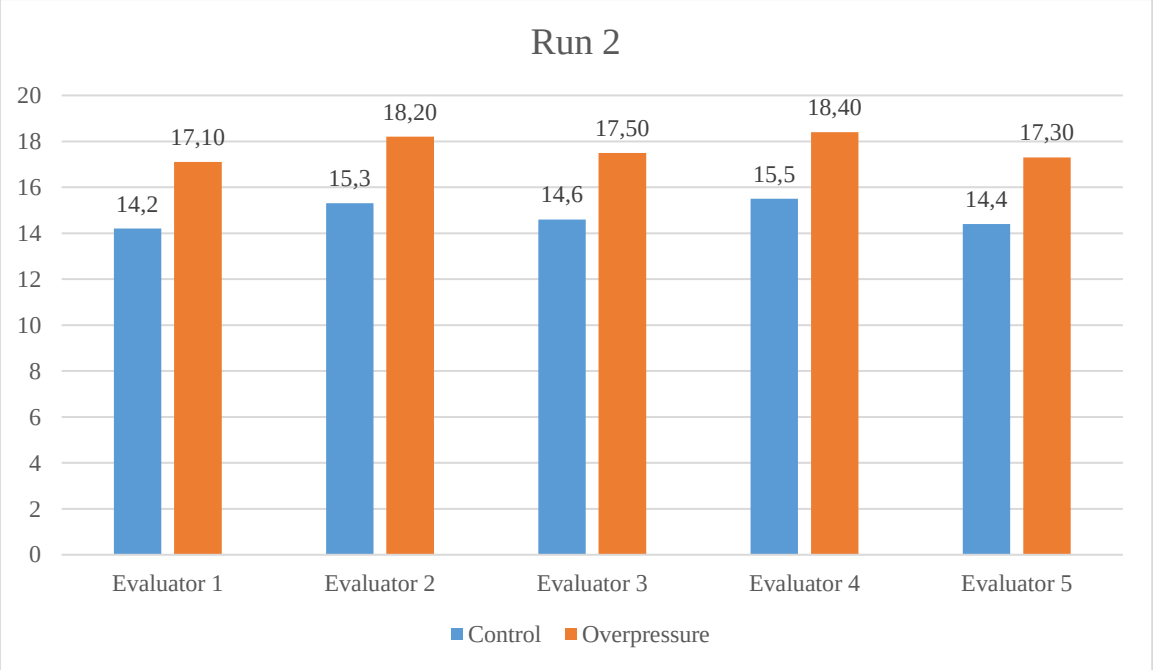


Figure 5. Run 2 – Individual sensory scores of cheese samples (overpressure vs. control)

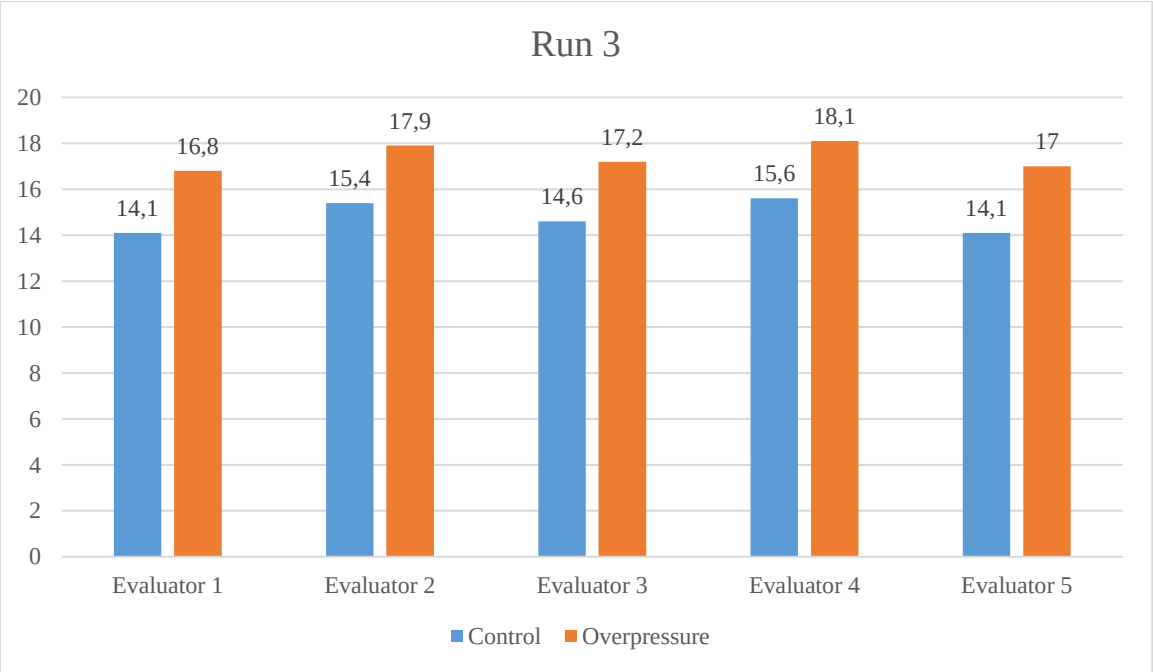


Figure 6. Run 3 – Individual sensory scores of cheese samples (overpressure vs. control)

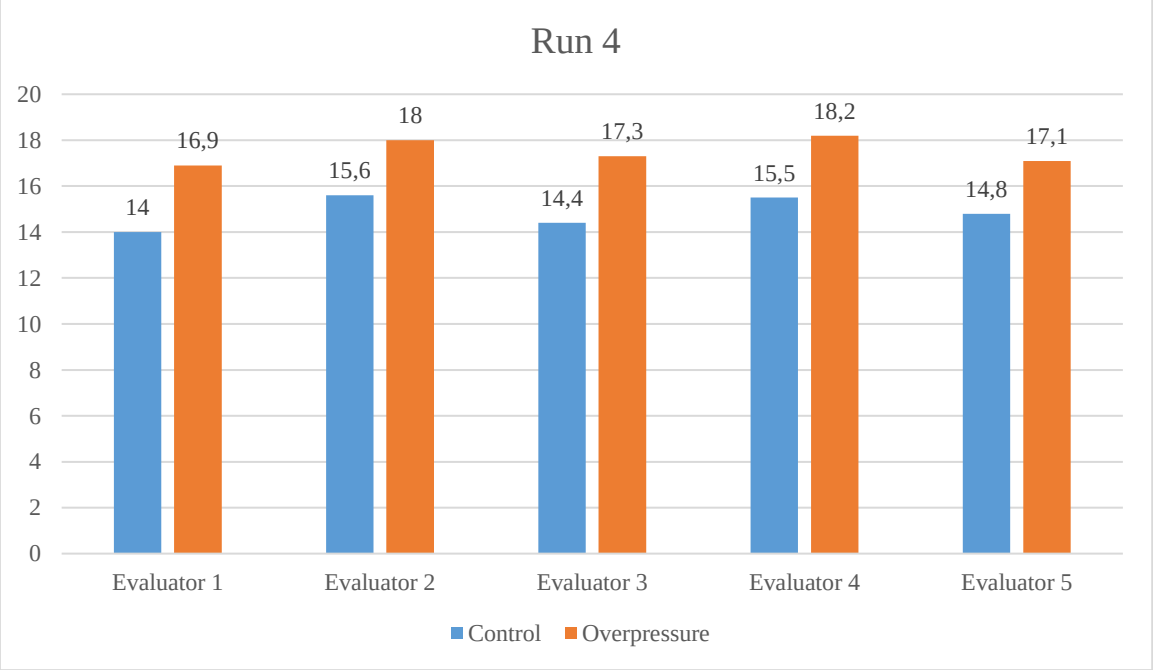


Figure 7. Run 4 – Individual sensory scores of cheese samples (overpressure vs. control)

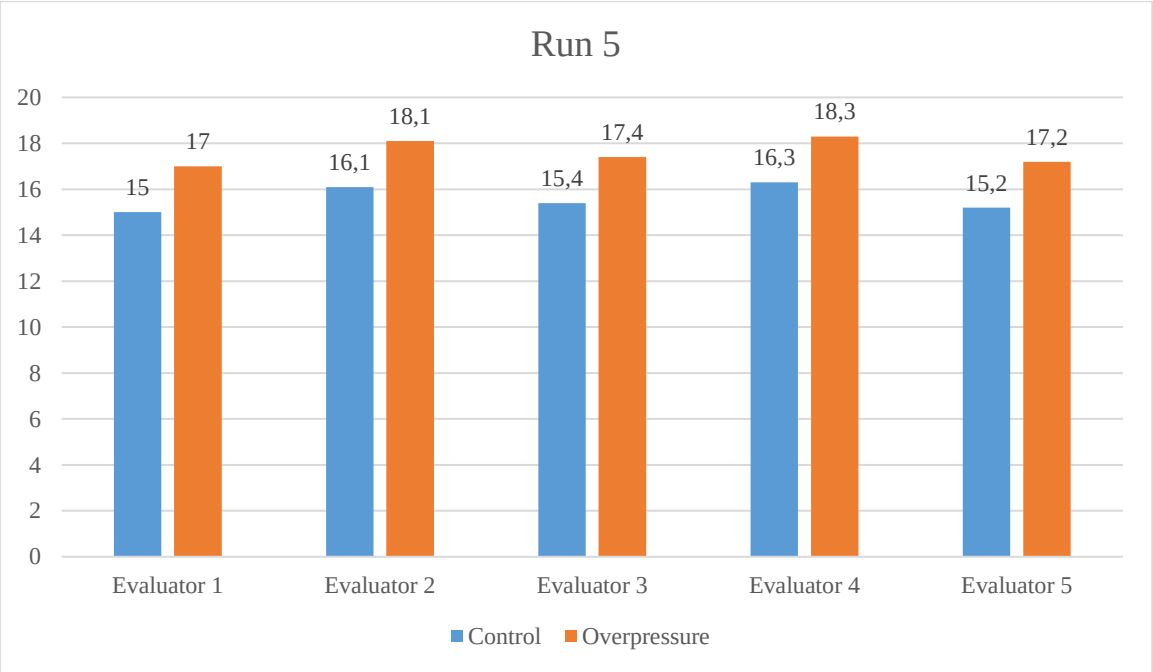


Figure 8. Run 5 – Individual sensory scores of cheese samples (overpressure vs. control)

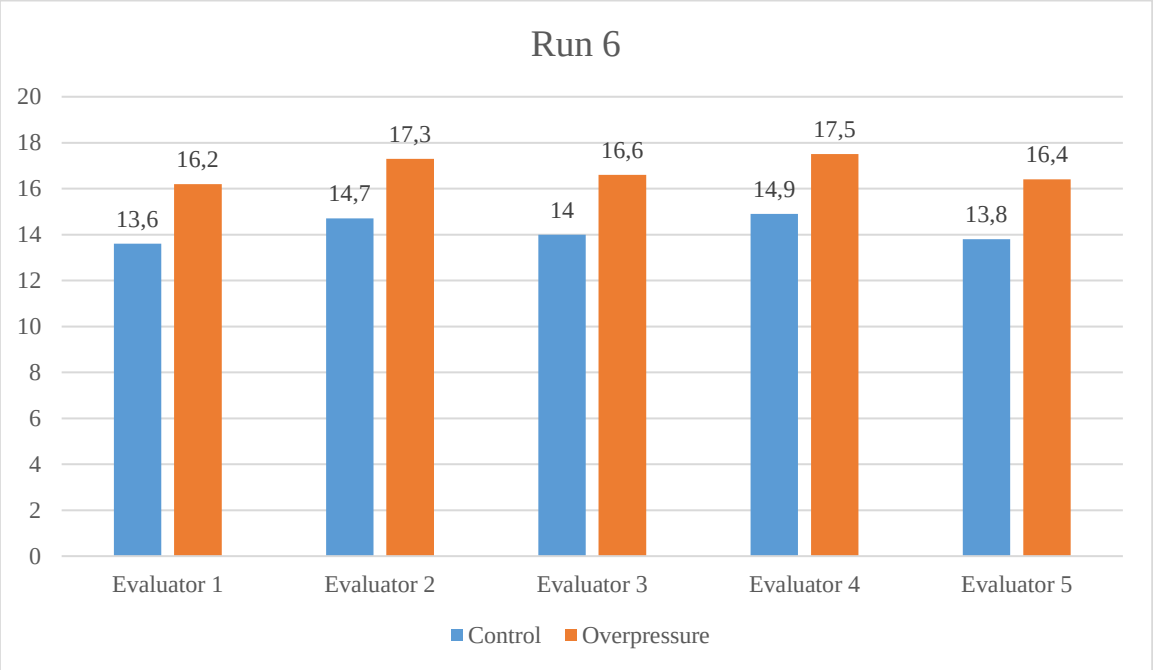


Figure 9. Run 6 – Individual sensory scores of cheese samples (overpressure vs. control)

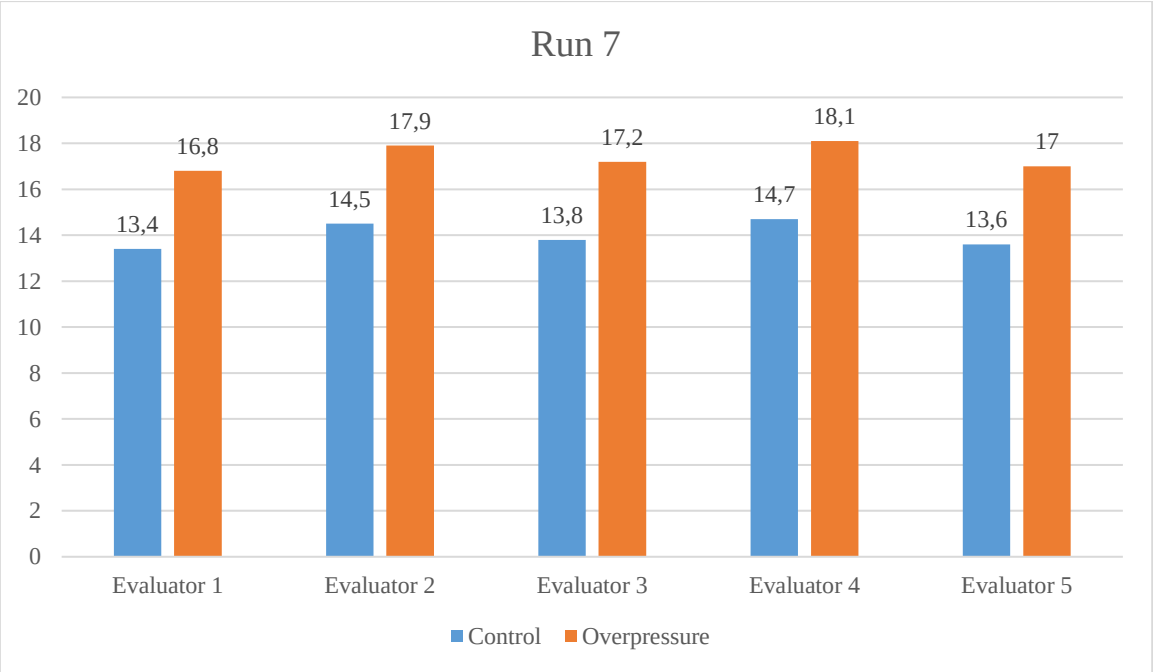


Figure 10. Run 7 – Individual sensory scores of cheese samples (overpressure vs. control)

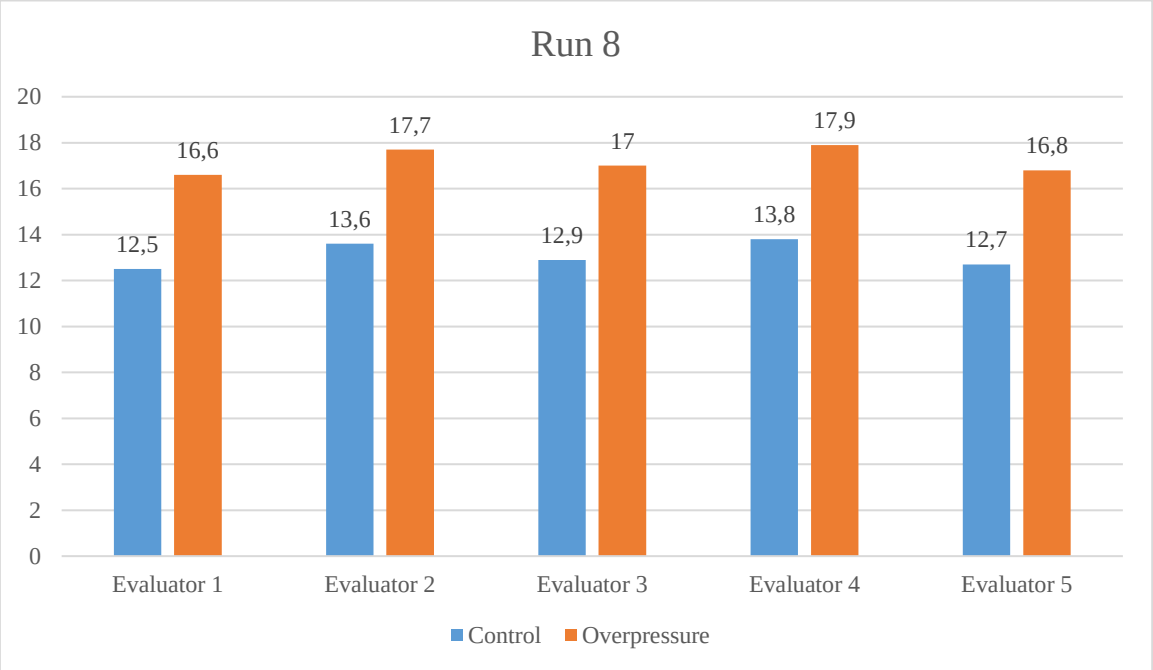


Figure 11. Run 8 – Individual sensory scores of cheese samples (overpressure vs. control)

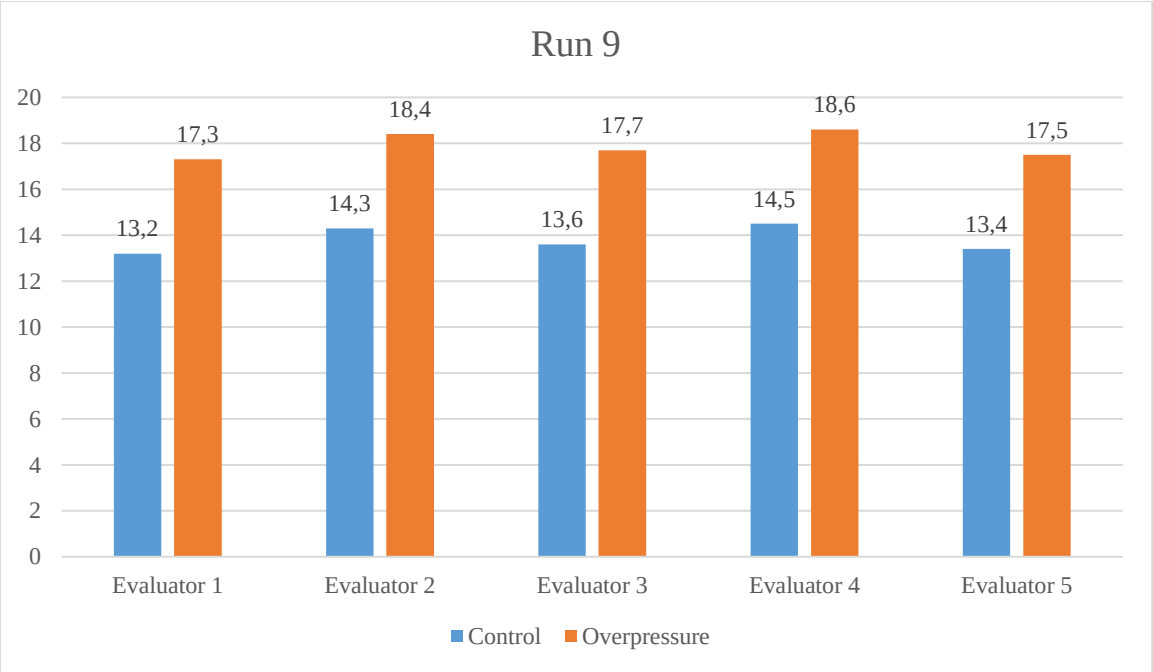


Figure 12. Run 9 – Individual sensory scores of cheese samples (overpressure vs. control)

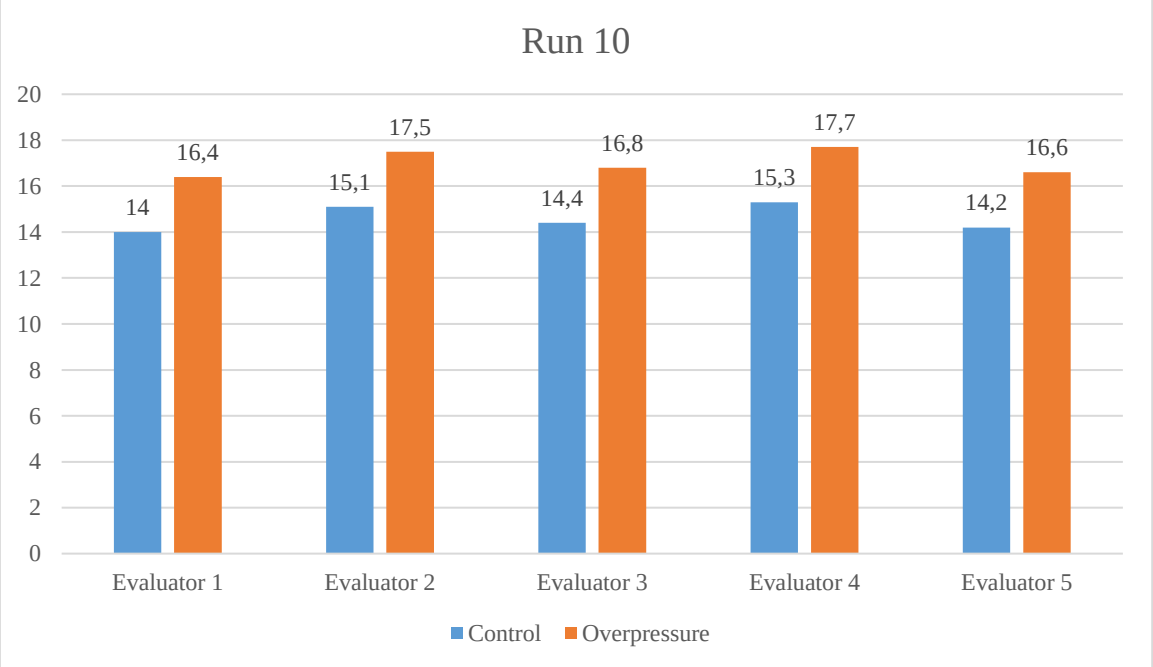


Figure 13. Run 10 – Individual sensory scores of cheese samples (overpressure vs. control)

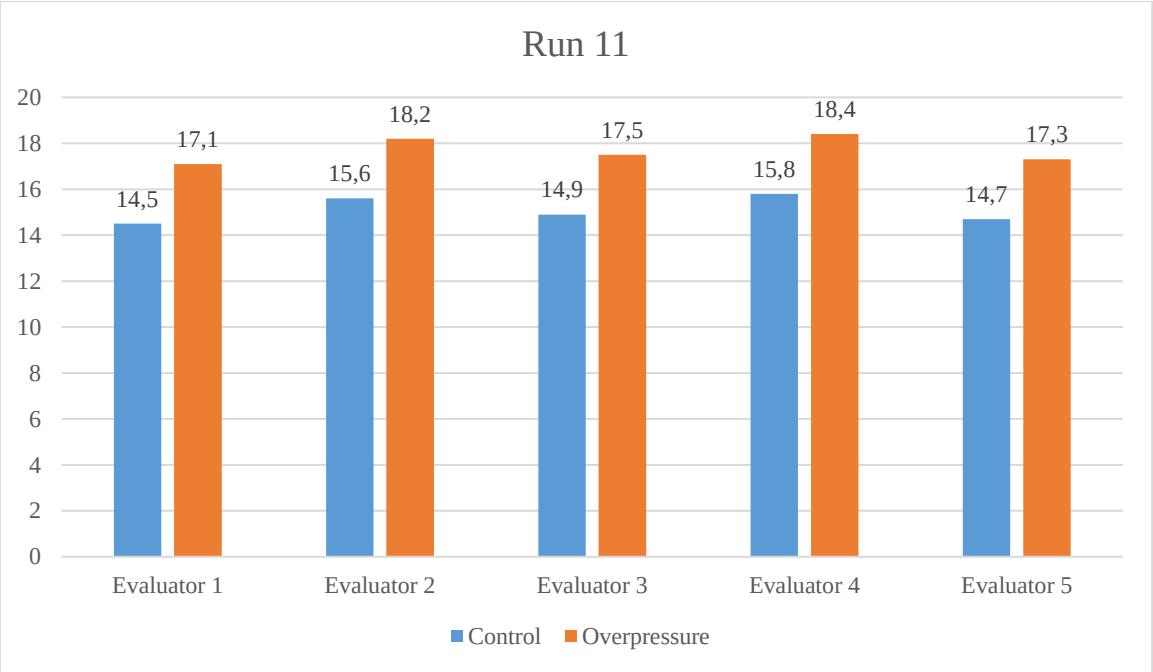


Figure 14. Run 11 – Individual sensory scores of cheese samples (overpressure vs. control)

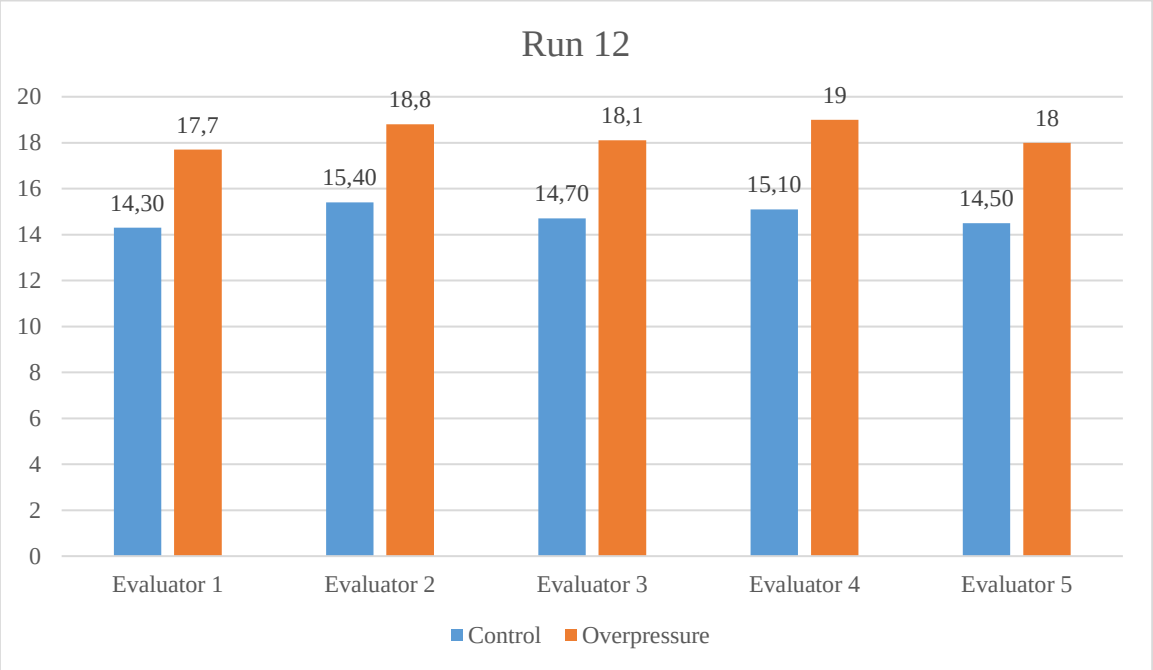


Figure 15. Run 12 – Individual sensory scores of cheese samples (overpressure vs. control)

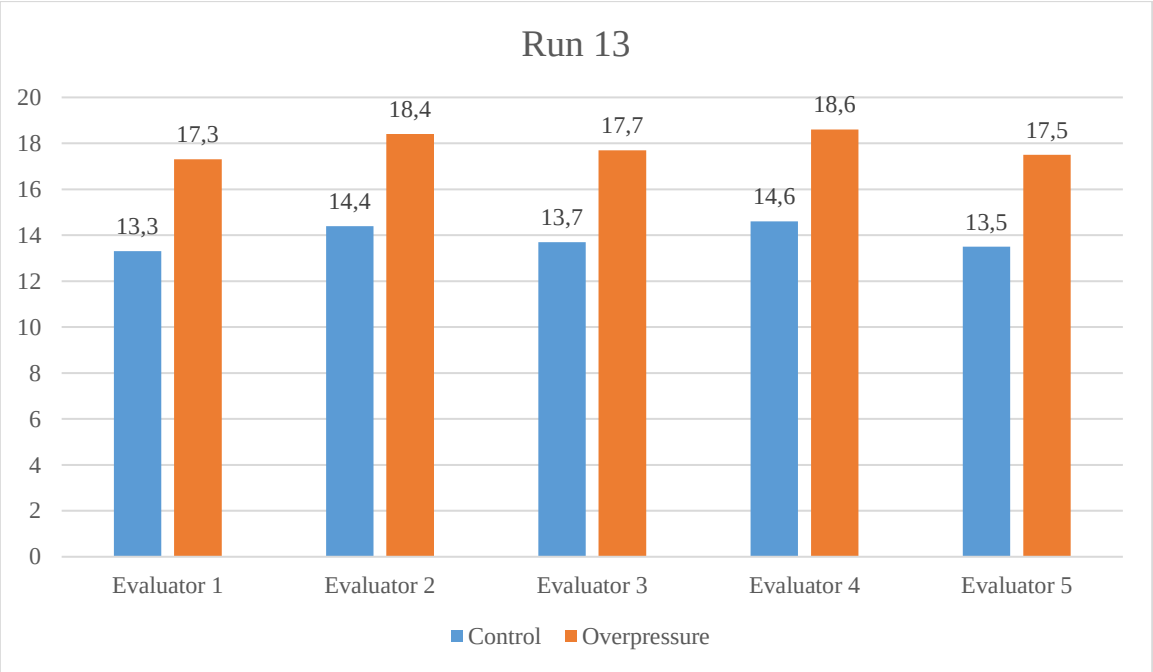
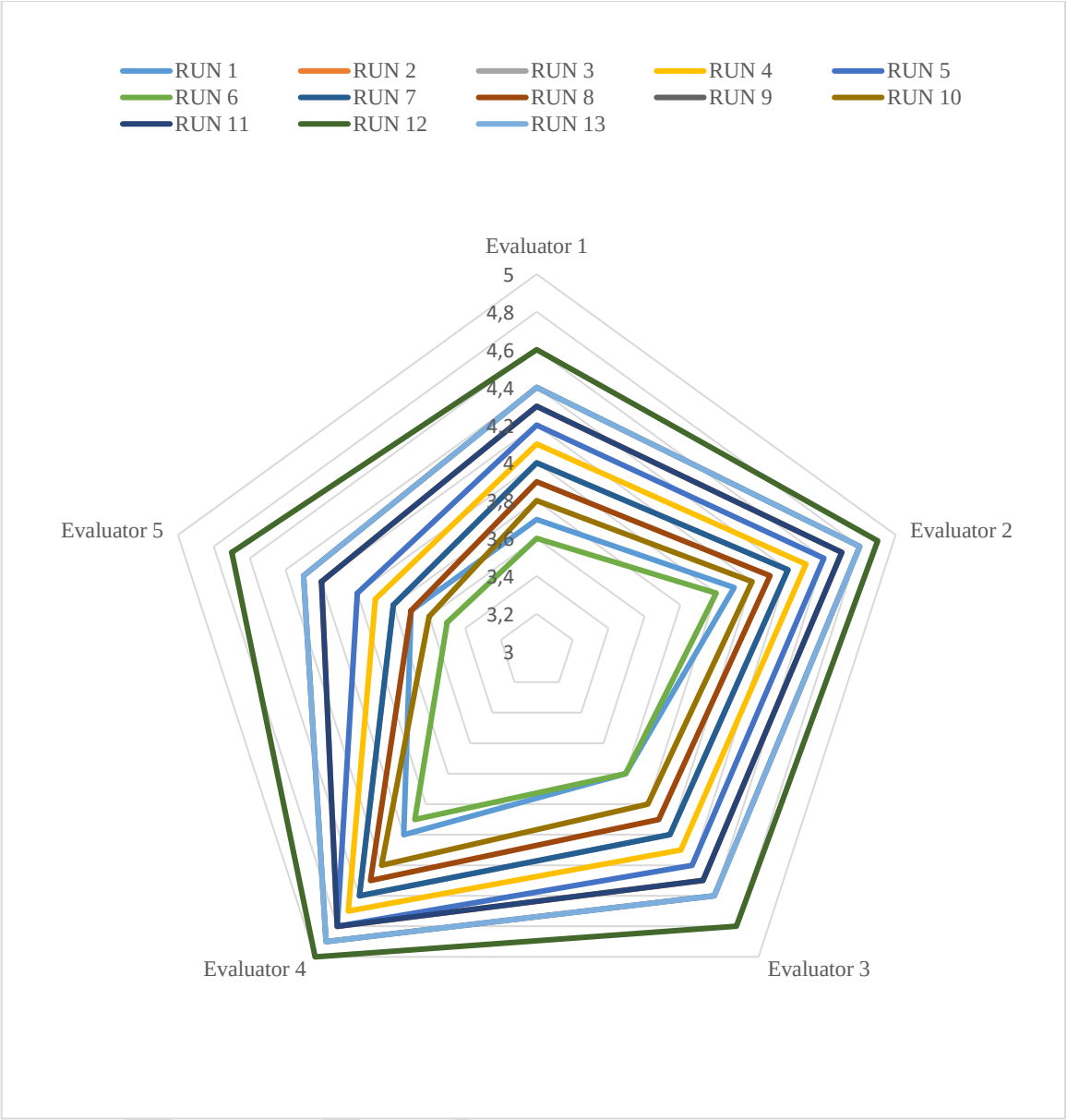


Figure 16. Run 13 – Individual sensory scores of cheese samples (overpressure vs. control)

Given that aroma is one of the most influential attributes contributing to overall sensory perception and consumer acceptance of cheese, a more detailed analysis of aroma evaluation was performed. Individual aroma scores assigned by the panellists (on a 1 to 5 scale) were examined to assess both the consistency of panel responses and the relationship between aroma perception and the overall sensory scores reported for each cheese sample. Among the evaluated samples, Run 12 achieved the highest mean aroma score (4.8), reflecting its pronounced and well-developed aromatic profile, which was consistent with its highest overall sensory acceptability. In contrast, Run 6 exhibited the lowest average aroma score (3.8), indicating a less intense and less complex aroma, in line with its lower total sensory evaluation. This approach allows a clearer interpretation of how aroma intensity and quality contributed to the observed differences in total sensory acceptability among the evaluated samples. The individual aroma evaluation results for each cheese sample are presented in **Figure 17**.

Figure 17. Individual aroma scores of cheese samples assigned by evaluators



The present study provides a comprehensive examination of how an overpressure environment during cheese ripening affects flavour development and microbiological quality, compared to atmospheric ripening. By employing a specially designed pressurized ripening chamber and analysing volatile compounds alongside microbial counts, the research offers novel insights into the cheese maturation process under non-traditional conditions. Overall, the findings indicate that even a moderate overpressure (on the order of a few millibars above atmospheric pressure) can significantly influence the cheese's aroma profile and microbial ecology. Key aroma compounds displayed distinct concentration changes under pressure, revealing shifts in metabolic pathways, while microbial analyses showed suppression of undesirable yeasts and altered mould growth dynamics. Importantly, the use of Response Surface Methodology (RSM)

enabled the modelling of these effects and the identification of optimal ripening conditions, highlighting overpressure as a dominant factor in volatile production. In the following, the main findings are synthesized and interpreted, focusing on aroma compound profiles, the role of specific fatty acids and lactones, microbiological outcomes, practical implications for cheesemakers, and directions for future research and innovation.

5. DISCUSSION

5.1. Microbiological Quality

A key concern for any cheese ripening process is the microbiological safety and quality of the product. The study addressed this by monitoring the presence of pathogens as well as the levels of yeast and mould in cheeses aged under normal and overpressure conditions. The results showed that pathogenic bacteria were consistently absent in all cheese samples throughout ripening time, which speaks to the good hygienic quality of the milk and the effectiveness of the process controls. Total viable bacterial counts remained within acceptable limits as well, indicating that the starter cultures and benign ripening microflora were thriving as expected, without any excessive growth of spoilers.

Focusing on yeasts and moulds, which are the primary spoilage organisms in aged cheeses, the study found that their levels were generally low in both control and overpressure cheeses (typically below 2.0 log CFU/g) at all sampling times. This suggests that the controlled ripening conditions (temperature, humidity, and overpressure) were effective in keeping yeast growth in check. In fact, yeasts were effectively suppressed under the overpressure conditions, the counts remained under the detectable threshold (<2 log) for most of the ripening, whereas the control chamber showed some higher yeast counts. For example, in week 4 and 5 the control had >4 log and 4.08 log CFU/g of yeast, whereas the 2.25 mbar chamber stayed <2.00 log at those times. This indicates a clear benefit of overpressure as unwanted yeast spread was reduced, likely preventing spoilage and off-flavours. The slight overpressure might have put yeasts at a disadvantage or limited their oxygen access, thus protecting the cheese from yeast spoilage. Additionally, the daily turning of the cheeses and the lack of rind washing in this experiment meant that no mould-inhibiting treatments were used, yet yeast levels remained low, highlighting that the environmental conditions alone (possibly combined with the dominance of a healthy starter culture) were enough to keep it under control.

The behaviour of moulds under pressure was a bit more complex. Moulds showed varying trends depending on the pressure level. In the overpressure chamber set to 2.25 mbar, mould counts initially increased during the first 3 weeks of ripening (reaching about 3.84 log CFU/g by week 3), then declined by week 5–7 down to 2.18–3.15 log. A similar pattern was observed at 3.0 mbar; an initial rise (mould counts in week 3 at 3.88 log, comparable to control 3.95 log), followed by a decline to <2.00 log by week 6 in the overpressure case (whereas the control at week 6 had 2.48 log, and by week 7 both were around 3–3.5 log). These data suggest that moulds can grow in the early phase of ripening even under elevated pressure, likely because at the start there are sufficient oxygen and nutrients available for spores to germinate and

mycelium to form. However, as the pressurization continues, the environment becomes increasingly unfavourable for mould survival or sporulation, leading to a drop in their counts after the initial peak.

Several factors could explain the pressure-induced inhibition of moulds in the later stages. Elevated pressure may reduce gas exchange, lowering the oxygen level in the chamber or on the cheese surface over time. Since moulds are aerobic, limited oxygen would hinder their growth once the initial supply is consumed. Increased pressure can also lead to higher levels of dissolved carbon dioxide in the cheese or surrounding atmosphere, and because carbon dioxide inhibits mould and fungal growth, its buildup in a sealed pressurized environment could act as a fungistatic agent. Additionally, starter bacteria may have outcompeted moulds for nutrients, particularly if bacterial metabolism increased under pressure, producing more volatiles and acidifying the cheese. This process reduces lactose and other nutrients available to moulds. Finally, the accumulation of fermentation by-products such as fatty acids, which often have antimicrobial properties, and a drop in pH could further suppress mould growth by weeks three to five, when these inhibitory conditions become more pronounced.

From a safety and quality standpoint, these microbiological findings are significant. They demonstrate that slight overpressure ripening can be used without elevating the risk of pathogenic contamination. The fact that *Listeria monocytogenes* and *Salmonella* spp. were consistently absent throughout the experiment shows that the process was hygienically designed and that the slight pressure may have helped create conditions unsuitable for these pathogens. However, it is important to note that pasteurized milk was used, so these bacteria were most likely never present in the first place.

The suppression of wild yeast is beneficial because it prevents the development of off-flavours. The controlled mould activity also shows that it might be possible to ripen cheese without applying surface treatments, yet still avoid heavy mould growth, as long as a bit of overpressure is used to keep it under control after the initial phase. In this way, the mild overpressure works like a gentle preservation method, not strong enough to kill or completely stop all microbes, as in high-pressure processing, but sufficient to give an advantage to the beneficial ones, like starter cultures and certain flavour-producing bacteria, while slowing down the spoilage organisms. This supports the idea that controlled ripening conditions play a key role in managing yeast and mould growth.

For cheesemakers, this could reduce the need for fungicides or anti-mould coatings on certain types of cheese, since the environment itself would help limit unwanted growth. However, in

cheeses where mould is essential for flavour and texture, like Camembert or blue cheeses, this approach might actually interfere with the desired mould development. Therefore, the method seems better suited for cheeses that should have a clean, mould-free rind or for those ripened from the inside, where mould is seen as contamination rather than part of the product.

Overall, the results show that ripening under mild pressure can maintain or even improve the microbial quality of cheese. By keeping yeast levels low and mould growth under control, this method helps prevent spoilage and defects during long aging periods. Combined with good hygiene and careful management of starter cultures, overpressure could make cheesemaking less dependent on preservatives and manual interventions, such as frequent rind treatments. These findings highlight how adjusting physical factors like pressure can shape both the flavour and the microbial balance of cheese, ultimately improving its safety and shelf life.

5.2. Aroma Compound Profiles

One of the central outcomes of this study is that cheese ripened under slight overpressure develops a different volatile aroma profile than cheese ripened under normal atmospheric conditions. Gas chromatography–mass spectrometry (GC–MS) analysis of headspace volatiles revealed that certain flavour compounds were either unique to or more abundant in the overpressure-ripened cheese, whereas others were found at higher levels in the conventionally ripened control. In particular, the comparison of volatile profiles showed that even a moderate increase in pressure during ripening shifts the metabolic pathways of flavour generation: the pressurized environment tended to enhance the formation of some volatiles while suppressing others. For example, compounds like 2-nonanone and benzothiazole were detected in the control cheese but were absent under overpressure conditions, suggesting that the pathways or microbial activities producing these molecules were inhibited by the elevated pressure. On the other hand, several fatty acids and lactones (discussed in detail below) were present at significantly higher concentrations in overpressure-ripened cheese, indicating that pressure stimulated their production or retention. Such differences imply that the organoleptic profile of the cheese can be tuned by adjusting the ripening pressure.

The mechanism behind these profile shifts likely involves the complex interplay of microbial metabolism and enzymatic activity under altered physical conditions. Even a mild overpressure can change the solubility of gases, the diffusion of metabolites, and the physiology of ripening microbes. In this study, the overpressure environment was tightly controlled in a chamber with regulated temperature, humidity, and airflow, ensuring that pressure was the primary variable. The data suggest that metabolic pathways were redirected under overpressure. For example,

pathways leading to certain ketones or sulphur-containing compounds may have been down-regulated, while pathways producing free fatty acids and lactone compounds were up-regulated. The result is a distinct aroma fingerprint for the pressurized cheese. Notably, even though the applied pressures were relatively low (approximately 1.014–1.017 bar absolute, i.e., 1.18–3.31 mbar above atmospheric), they were sufficient to measurably impact the ripening biochemistry. This finding underscores that cheese ripening is sensitive to environmental pressure in ways not previously recognized, given that traditional affinage rarely considers pressure as a factor.

From a sensory perspective, the differences in volatile profiles could translate to perceptible differences in flavour. For instance, 2-nonanone (prominent in control but absent under pressure) is known to impart blue-cheese or fruity notes in some cheeses, whereas the fatty acids that increased under pressure contribute piquant, rancid, or goaty notes when above certain thresholds. The lactones enhanced by pressure contribute sweet and creamy, coconut-like aromas. Thus, the overpressure-ripened cheese might skew toward a creamier, fatty-sweet aromatic profile with reduced pungent or musty notes. Such a profile could be desirable or not, depending on the cheese style and consumer preferences. Importantly, the study's comprehensive screening of volatiles provided a foundation for selecting key aroma-active compounds for quantitative analysis. By focusing on those key compounds, the researchers could clearly illustrate how controlled pressure and ripening time each influence the major aroma constituents.

Free fatty acids emerged as some of the most influential aroma compounds in this study, aligning with the well-known fact that free fatty acids are important, and often dominant contributors to cheese flavour development. Four saturated fatty acids in particular were highlighted: hexanoic (C6), octanoic (C8), decanoic (C10), and dodecanoic (C12) acids. These medium-chain fatty acids are typical products of lipolysis during cheese ripening and each has a distinct impact on aroma:

Hexanoic acid (caproic acid) is commonly associated with a sharp, goat or barnyard aroma in cheese, and in many cheese varieties (especially those made from goat's milk or aged for a long time) it contributes to the piquant flavour. In the present study, hexanoic acid was among the monitored responses and its concentration tended to increase with overpressure. The RSM analysis indicated that higher chamber pressure led to significantly higher hexanoic acid levels in the cheese. However, there was evidence that after a certain ripening duration (around 5–6 weeks), hexanoic acid concentrations plateaued or even declined slightly. This suggests there may be an optimal window during which hexanoic acid peaks, after which it might volatilize

or be metabolized into other compounds. The elevated levels of hexanoic acid under pressure could enhance the tangy, aged flavour of the cheese, but excessive accumulation might risk an overly pungent or rancid note if not controlled.

Octanoic acid (caprylic acid) was strongly affected by the interplay of pressure and time. This C8 acid can impart rancid, oily, or goat aromas, and interestingly it also has known antimicrobial properties as caprylic acid is used as an inhibitor of certain bacteria and moulds. The study found a wide range of octanoic acid concentrations across the different ripening trials, from as low as 3.8% up to 25.99% of the total identified volatiles. Such variability underscores how sensitive octanoic acid formation is to ripening conditions. The highest octanoic acid levels were observed in cheeses that ripened for relatively short times under moderate to higher pressure (e.g., one run at 3.59 weeks and 2.25 mbar yielded the highest percentage of octanoic acid). This implies a synergistic effect: pressure combined with shorter ripening can dramatically boost octanoic acid production, perhaps by accelerating lipolysis or liberating fatty acids faster than they can be further metabolized. RSM confirmed a significant interaction between pressure and time for octanoic acid levels. Practically, this means that to maximize desirable notes from octanoic acid without overshooting into rancidity, one might use a higher pressure for a shorter period. However, caution is needed, as octanoic acid's potent odour can dominate if too concentrated. The antimicrobial aspect of octanoic (and other medium-chain fatty acids) also suggests that as these levels rise, they might help suppress spoilage microbes in the cheese matrix, which is an interesting side-benefit aligning with the microbiological observations.

n-decanoic acid (capric acid) was another critical fatty acid examined. Capric acid has a strong, rancid and sometimes "goaty" odour that is usually considered undesirable in excess. In fact, in many dairy products, high levels of n-decanoic acid are linked to off-flavours described as soapy or animal-like. This study found that higher overpressure and longer ripening times both tended to promote the formation of n-decanoic acid, whereas the lowest concentrations of n-decanoic acid were observed under the mildest pressure conditions (1.18–1.50 mbar). Statistical modelling corroborated that increasing either pressure or time significantly elevated n-decanoic acid levels (both factors had $p < 0.05$ for this response). There was also a notable pressure – time interaction for n-decanoic acid, similar to octanoic, meaning prolonged aging under high pressure particularly exacerbated n-decanoic acid accumulation. Interestingly, the *highest* concentrations of n-decanoic acid (and some other fatty acids) were obtained at the shortest ripening time and a mid-range overpressure (2.25 mbar). This somewhat suggests that pressure

can accelerate the enzymatic release of this fatty acid early in ripening, but if given more time, some n-decanoic acid might be further transformed or volatilized. In terms of flavour, the implication is that pressure can induce strong pungent notes quickly, so controlling the duration is key to avoid an accumulation of capric acid that could make the cheese unpleasant. The study notes that while *caproic, caprylic,* and *capric acids* are indeed found in various aged cheeses (cheddar, Grana Padano, and especially goat cheeses) and can contribute characteristic tanginess, they must be balanced, where a small amount may add piquancy, but too much is overwhelmingly rancid. Thus, the ability of the overpressure chamber to drive up n-decanoic acid levels is a double-edged sword as it could be harnessed for intensifying certain flavour notes in a short time frame, but it requires optimization (via tools like RSM) to prevent flavour defects.

Dodecanoic acid (lauric acid), a 12-carbon saturated fatty acid, was also quantified in the cheese volatiles. Lauric acid is known for imparting soapy or waxy off-flavors in dairy products. It is less common as a dominant note in cheese flavour, and generally, cheesemakers aim to keep its level low. In this study, dodecanoic acid was present at lower concentrations relative to the other major aroma compounds, on the order of 1.27% to 13.3% of the total identified volatiles depending on the sample. The fact that lauric acid remained at modest levels is considered favourable for overall flavour quality. The data indicated that overpressure did increase lauric acid content to some extent (higher pressures yielded more lauric acid, consistent with the trend for other fatty acids), but even at its peak it did not reach the intensity of octanoic or decanoic acids in any sample. This is beneficial, as even moderate amounts of lauric acid can be detected by consumers as an undesirable waxy note. Thus, one practical outcome is that overpressure ripening does not seem to drastically elevate lauric acid to problematic levels (it preferentially elevated shorter-chain acids more) which might mean the method intensifies certain flavours without incurring too much soapy character.

Mechanistically, the generation of these free fatty acids under pressure can be attributed to enhanced lipolytic activity or altered microbial dynamics. During normal cheese ripening, lipases (from lactic acid bacteria or milk) gradually hydrolyse milk fat triglycerides, releasing fatty acids over time. The presence of higher pressure might stress certain microbes or favour lipase-producing microorganisms, accelerating lipolysis early in the ripening period. Additionally, pressure may slow the volatilization of these fatty acids (by increasing the cheese matrix's retention of volatile compounds), effectively concentrating them in the cheese paste. The study's results, particularly the observation that the highest levels of several fatty acids

occurred at the shortest ripening time under moderate pressure, support the idea that pressure speeds up initial flavour release. Yet, as noted, beyond 5 to 6 weeks the levels of some acids began to level off or decline, which could mean that the system reaches a new equilibrium where microbes begin to metabolize the fatty acids into other products (like methyl ketones or lactones) or the acids slowly evaporate out of the cheese. This points to a nuanced conclusion: pressure can front-load the ripening process, generating a burst of flavour compounds early, but maintaining those compounds or continuing to increase them may require additional strategies or different pressures as ripening progresses.

Beyond free fatty acids, the study identified lactones as significant aroma compounds influenced by overpressure. Lactones are cyclic esters often derived from hydroxy fatty acids, and they are well-known for contributing creamy, fruity, and sweet notes to dairy product aromas. Two lactone compounds were of particular interest:

3,5-Dihydroxydecanoic acid 1,5-lactone is a lactone derived from a dihydroxy-decanoic acid, and was found to be the most abundant single aroma component in the cheese samples. Its predominance was significant under the special ripening conditions, suggesting that overpressure enhanced the pathways of certain microorganisms that produce this lactone. As earlier mentioned, the presence of 3,5-dihydroxydecanoic acid lactone can be influenced by fungal metabolism. The fact that this lactone was so abundant under overpressure conditions implies that mild overpressure did not inhibit fungal activity entirely, in contrast it stimulated certain metabolic pathways. This lactone gives a rich and fatty character with a slight sweetness to the cheese aroma without the need for actual dairy fat increase. In traditional cheese, lactones are often associated with buttery, peachy, or coconut aromas and are considered key to the aroma of many ripened cheeses. The higher levels of 3,5-dihydroxydecanoic lactone in this study points to a pressure enhancement in secondary lipid metabolism, possibly from cheese surface microflora or internal yeasts that survived pasteurization.

Delta-dodecalactone is a well-known lactone in dairy industry, often described as having a powerfully sweet, creamy, and fruity aroma reminiscent of peach, apricot, or coconut. In the study, delta-dodecalactone concentrations were found to increase under higher overpressure treatments (approximately 2.25–3.31 mbar). In cheeses ripened at the upper end of the pressure range, delta-dodecalactone was significantly elevated compared to those ripened at atmospheric pressure. The likely sensory impact is an enriched buttery-sweet aromatic profile in the high-pressure cheeses. Because delta-dodecalactone is considered a desirable aroma compound, its increase under pressure is a positive outcome. It suggests that overpressure ripening can amplify

the *creamy and caramel* notes of the cheese. Notably, delta-dodecalactone is not typically abundant in all cheeses, its presence may be associated with specific microbial actions. The controlled overpressure conditions might have created an environment favourable to lactone-producing microbes, or influenced the enzyme activities that convert fatty acids to lactones. The study's results strongly indicate that pressure was the dominant factor driving delta-dodecalactone formation, the highest levels were seen whenever pressure was high, regardless of time. Time had a smaller effect, suggesting that once the pressure threshold to activate these pathways is reached, the production of delta-dodecalactone proceeds effectively within the ripening period examined.

In addition to these, other compounds, such as benzoic acid, were detected. However, the fatty acids and lactones were the primary focus because of their abundance and aroma impact. The connection between fatty acids and lactones is also interesting as many lactones in cheese arise from fatty acid precursors. The increase in free fatty acids (as noted above) could provide more substrate for lactone formation, as study shows, the high levels of 3,5-dihydroxydecanoic lactone might be traced to the availability of decanoic acid and its derivatives under pressure. Thus, overpressure not only increases certain fatty acids but may subsequently drive secondary reactions (like lactonization) that yield these sweet-aroma compounds.

As is well-known, free fatty acids being important, and often dominant, components of cheese flavour. The concentration of aroma compounds in cheese during ripening results from a complex interaction between pressure, time, and other cheese-specific ripening parameters. While higher pressures may increase the concentration of certain compounds and longer ripening times can influence the levels of some aroma compounds, the relationship is not uniform across all compounds. According to GC/MS analysis, the optimal conditions for cheese ripening in an overpressure chamber, along with the best microbiological quality, involve shorter ripening times and overpressure conditions of 3.00 mbar. However, the data indicates that the concentration of aroma compounds can vary considerably between cheese samples, even under the same pressure and time conditions. It should be noted that variability was observed in aroma composition even for cheeses treated under nominally identical pressure and time conditions. This suggests that cheese ripening is affected by additional factors, including specific microbial and enzymatic activities, complex biochemical processes, the cheese production process, cheese handling, and the cheese's position in the ripening chamber, among others. In practice, two cheeses ripened side by side in the same chamber could have significantly different concentrations of certain volatiles. Based on the analysed data, it can be

concluded that the ripening process has a significant impact on the presence of yeast and mould in cheese samples. Controlled ripening conditions seem to effectively suppress yeast growth, while variations in mould presence depend on different ripening conditions and pressure levels. This emphasizes the role of micro-environmental differences and microbial colony variability: slight differences in the distribution of microorganisms, or microclimates of pH/moisture on the cheese surface, can lead to divergent outcomes. Further studies may be necessary to gain a deeper understanding of the mechanisms driving these changes and to optimize ripening conditions for improved microbiological quality control.

5.3. Physicochemical Composition

The physicochemical composition of the experimental cheeses, fat, moisture, protein, and salt content, remained throughout the study within the limits prescribed for semi-hard cheeses under Croatian and international regulatory frameworks (*Pravilnik o sirevima, NN 20/2009*), confirming that neither the application of continuous overpressure nor the variation in ripening duration compromised the legal identity or nutritional integrity of the product. This finding is of practical relevance, as it establishes the technological feasibility of overpressure-assisted ripening as a process modification compatible with standard product classification requirements.

Fat content across the experimental runs followed a pattern consistent with the well-established concentration mechanism operative during cheese maturation: as moisture is progressively lost from the matrix through evaporation, the relative proportions of the retained solid constituents, such as fat, increase accordingly. The observed inverse relationship between fat and moisture content is therefore not attributable to any direct pressure-mediated effect on the lipid fraction itself but rather reflects the extent to which overpressure and ripening duration collectively governed the dehydration of the cheese matrix. The overall range of fat values recorded across the experimental matrix falls within the interval typically reported for semi-hard cheeses manufactured from standardised cow's milk, and at no point approached a threshold that would compromise sensory or nutritional quality. This finding indicates that overpressure, at the levels applied in the present study, does not alter the lipid fraction through mechanisms independent of moisture redistribution.

Moisture content emerged as the parameter most sensitive to both process variables and, consequently, as the primary driver of compositional variation across the experimental matrix. The mechanistic basis for the observed association between higher overpressure and greater moisture retention lies in the physical effect of positive air pressure on the vapour pressure

gradient at the cheese surface. Sustained overpressure reduces the differential between the water vapour pressure of the cheese and that of the surrounding atmosphere, thereby attenuating evaporative moisture loss during the ripening period. This mechanism represents a meaningful distinction from the effects of high-pressure processing applied as a short-duration, high-intensity treatment, where compression typically promotes whey expulsion and accelerates moisture reduction. The continuous, low-level overpressure employed in the present study thus appears to operate through a fundamentally different physical pathway, one that moderates rather than enforces dehydration , and should not be interpreted through the same mechanistic lens as conventional high pressure processing.

Protein content mirrored the fat content pattern in its inverse relationship with moisture, reflecting the same concentration-driven mechanism. Beyond this dilution effect, however, the variation in protein content across runs also likely reflects differences in the extent of proteolysis under the different ripening conditions. Proteolytic breakdown of casein fractions generates soluble peptides and free amino acids, a proportion of which are lost with expressed whey or evaporated moisture, thereby reducing the total detectable protein in the final product. The higher protein values observed in runs characterised by lower overpressure and longer ripening duration suggest either less extensive proteolysis or more effective retention of the soluble nitrogen fraction within a denser, lower-moisture matrix. This interpretation is supported by the GC–MS volatile compound data, in which higher-pressure conditions were associated with elevated concentrations of volatiles derived from proteolytic and amino acid catabolism pathways, implying that the more hydrated matrices produced under elevated overpressure also sustained greater proteolytic metabolic activity.

Salt content showed a pattern most directly explained by variation in moisture content, as the absolute mass of sodium chloride absorbed during brining is approximately constant across batches of equivalent size and brine exposure. Consequently, a reduction in moisture raises the salt-in-moisture (S/M) ratio even in the absence of any change in total salt uptake, while a more hydrated matrix facilitates greater salt diffusion from the surface brine into the cheese interior. The calculated S/M ratios across the experimental matrix span a range at which proteolytic and lipolytic enzyme activities are known to be progressively inhibited, meaning that differential salt-mediated enzyme regulation across runs constitutes a plausible additional mechanism contributing to the observed variability in volatile aroma compound profiles. This enzymatic modulation by salt is therefore an important contextualising factor that must be considered alongside overpressure and ripening time when interpreting the GC–MS results, as it introduces

a further dimension of biochemical regulation not fully captured by either process variable independently.

5.4. Sensory Quality

The results of the sensory analysis demonstrated a clear distinction in flavour and aroma development between cheeses matured under overpressure and those ripened at atmospheric pressure. Cheeses subjected to pressurized conditions were consistently rated higher in overall acceptability, aroma intensity, and flavour complexity. In particular, samples exhibiting the highest overall sensory scores also achieved the highest mean aroma ratings, confirming the central role of aroma in shaping overall flavour perception and consumer acceptability. These findings indicate that mild overpressure during ripening can positively influence the sensory quality of semi-hard cheese.

Importantly, the cheeses that received the highest sensory scores and aroma scores also showed elevated concentrations of desirable volatile aroma compounds, including octanoic, decanoic, and dodecanoic acids, as well as 3,5-dihydroxydecanoic acid and δ-dodecalactone. These compounds are known contributors to creamy, buttery, and fruity aroma notes. This alignment between analytical and sensory results suggests a strong correlation between the volatile profile and the perceived sensory quality of the cheeses. The presence and balance of specific medium-chain fatty acids and lactones appear to be key drivers of aroma intensity and flavour enhancement, particularly under modified ripening conditions. The study thus supports the use of controlled overpressure as a promising strategy for improving the sensory properties of cheese through targeted biochemical modulation.

5.5. Practical Implications and Scientific Contribution

This research offers valuable practical insights for the dairy industry, particularly for cheesemakers seeking to innovate or refine ripening processes. Ripening under overpressure conditions can accelerate flavour development, allowing cheeses to reach a desired flavour intensity in four to five weeks instead of the usual six or more. This shorter ripening time can reduce storage costs, minimize weight loss, and improve yield, while still producing flavour profiles that match or even surpass traditional methods. Overpressure can also help cheesemakers fine tune flavour characteristics by influencing microbial metabolism and volatile compound formation.

From a quality and safety standpoint, overpressure ripening reduces spoilage by limiting the growth of unwanted yeasts and moulds, extending shelf-life and improving consistency. The

process may also lower the need for fungicides or other anti-mould treatments, as the slightly pressurised environment naturally discourages contamination. Because the cheeses in the study required no surface treatments and still remained free of excessive mould, producers could adopt a more automated, low-intervention approach to ripening, reducing labour and contamination risks. While specialised equipment is necessary, the pressures involved are minimal, so energy and structural demands are low compared to conventional high-pressure systems. However, scaling up from pilot to industrial levels will require careful design to ensure even pressure distribution and consistent ripening across larger volumes.

Not all cheese types may benefit equally from this method. Semi-hard and hard cheeses such as Cheddar, Gouda, or Edam appear most suitable, while mould-ripened or long-aged cheeses may not respond as positively, since their characteristic microflora or textures depend on surface moulds or extended protein crystallisation. Regulatory and consumer considerations are also important. Because overpressure ripening does not alter cheese composition or introduce additives, it should be acceptable within current regulations. In fact, "pressure-ripened" cheeses could become a distinctive product category, potentially appealing to consumers if the flavour and texture align with expectations.

Future research should explore how different microbial strains behave under overpressure, how pressure affects enzyme activity and proteolysis, and whether results can be replicated in larger or different types of cheese. Further studies could also assess sensory outcomes and economic feasibility to determine whether the benefits outweigh the investment in equipment. From a scientific perspective, this study contributes to a new understanding of how physical conditions shape biochemical and microbial dynamics during ripening. Unlike traditional high-pressure processing, which focuses on sterilisation, continuous mild pressure subtly directs flavour development and microbial balance. Overall, the findings demonstrate that slight overpressure during ripening can be a powerful yet simple tool for improving cheese quality. It offers faster flavour development, greater control over microbial populations, and potentially lower costs, all while aligning with clean-label and sustainability goals. This approach expands the cheesemaker's toolkit, showing that even a small physical adjustment, pressure, can meaningfully influence the safety, flavour, and efficiency of cheese production, marking a promising step forward in dairy processing innovation.

6. CONCLUSION

Based on the complete set of experimental results obtained in this doctoral research, encompassing microbiological characterisation, GC–MS volatile compound profiling, physicochemical composition analysis, and sensory evaluation, the following conclusions are drawn:

1. Microbiological analyses confirmed the complete absence of *Salmonella* spp., *Listeria monocytogenes*, and Coagulase positive *Staphylococcus* in all samples throughout the ripening period. Yeast counts were consistently suppressed under overpressure, while mould populations exhibited a characteristic biphasic pattern, an initial increase during the first 2 to 3 weeks followed by a progressive decline, indicating that sustained overpressure ultimately limits mould proliferation without eliminating the early mould activity associated with lactone production.
2. The volatile aroma profile of semi-hard cheese is significantly influenced by the pressure of the ripening environment. Even a moderate overpressure of 1.18 – 3.31 mbar above atmospheric was sufficient to shift metabolic pathways during ripening, suppressing certain compounds, such as 2-nonanone and benzothiazole, while enhancing others. Of 118 volatile compounds identified by GC–MS, 67 were present in only one treatment group, confirming that overpressure induces a qualitatively distinct aroma fingerprint.
3. 3,5-Dihydroxydecanoic acid 1.5-lactone was the most abundant single aroma compound in the overpressure-ripened cheese, indicating that the pressurised ripening environment selectively enhanced the activity of lactone-producing microorganisms. Delta-dodecalactone was also significantly elevated at higher pressure treatments. The preferential enrichment of lactone compounds, associated with creamy, sweet, fruity, and coconut-like sensory notes, represents the most technologically relevant outcome of the overpressure ripening approach.
4. Overpressure was the dominant factor governing the formation of all six RSM-modelled volatile responses, as confirmed by statistically significant linear pressure coefficients for all compounds. Ripening time exerted a secondary and selective influence, reaching statistical significance only for octanoic acid and n-decanoic acid, for which a significant pressure–time interaction was also observed, underscoring the importance of coordinated parameter optimisation for these two fatty acids in particular.

5. RSM modelling identified the optimal ripening conditions as approximately 3.00 mbar overpressure and 5.0 – 5.5 weeks of maturation, under which desirable lactone compounds were maximised, free fatty acids were maintained at balanced levels, and microbiological quality was optimal. This optimised parameter set is directly applicable as a process recommendation for cheesemakers adopting overpressure ripening technology.
6. All physicochemical parameters assessed, fat, moisture, protein, and salt content, remained within the prescribed standards for semi-hard cheese throughout the study, confirming that overpressure-assisted ripening does not compromise the legal identity or nutritional integrity of the product. Moisture content proved the most responsive parameter, with higher overpressure retarding evaporative water loss and thereby indirectly modulating the salt-in-moisture ratio and the activity of proteolytic and lipolytic enzymes, effects that were ultimately reflected in the volatile aroma compound profile.
7. Sensory evaluation demonstrated that overpressure-ripened cheeses achieved substantially higher total sensory scores compared to control samples ripened at atmospheric pressure. The highest overall sensory score and the highest mean aroma score were recorded for cheese ripened at 2.25 mbar for 5 weeks, and the narrow score distribution among cheeses produced under identical conditions confirms the reproducibility of the overpressure ripening process.
8. The findings of this doctoral research collectively demonstrate that continuous mild overpressure represents a technologically feasible, chemically clean, and sensorially effective strategy for improving semi-hard cheese quality. By simultaneously promoting the formation of desirable aroma compounds, suppressing spoilage microorganisms, and preserving compositional integrity, overpressure-assisted ripening offers the dairy industry a novel and scalable tool for achieving faster, more consistent, and higher-quality cheese maturation, without recourse to additives, preservatives, or intensive manual intervention.

7. REFERENCES

Altinoz, M. A., Ozpinar, A. and Seyfried, T. N. (2020) Caprylic (octanoic) acid as a potential fatty acid chemotherapeutic for glioblastoma. Prostaglandins, Leukotrienes and Essential Fatty Acids, 159, 102142. https://doi.org/10.1016/j.plefa.2020.102142

Alves, A. C. de O. and Pérez, M. H. (2020) Study of the effect of high hydrostatic pressure in the decrease of Listeria monocytogenes in pasteurized mixed cheese. Brazilian Journal of Development, 6(5), pp. 23242–23252. https://doi.org/10.34117/bjdv6n5-032

Anelli, P., Haidukowski, M., Epifani, F., Cimmarusti, T. M., Moretti, A., Logrieco, A. and Susca, A. (2018) Fungal mycobiota and mycotoxin risk for traditional artisan Italian cave cheese. Food Microbiology. https://doi.org/10.1016/j.fm.2018.09.014

Atallah, A. A., El-Deeb, A. M. and Mohamed, E. N. (2021) Shelf-life of Domiati cheese under modified atmosphere packaging. Journal of Dairy Science, 104(8), pp. 8568–8581.

Bachmann, H.-P. (2011) Cheese – Swiss-type cheeses. In: Fuquay, J. W. (ed.) Encyclopedia of Dairy Sciences. 2nd edn. Amsterdam: Elsevier, pp. 712–720. https://doi.org/10.1016/B978-0-12-374407-4.00085-6

Bas, D. and Boyaci, I. H. (2007) Modelling and optimization I: Usability of response surface methodology. Journal of Food Engineering, 78(3), pp. 836–845. https://doi.org/10.1016/j.jfoodeng.2005.11.024

Bäumlisberger, M., Moellecken, U., König, H. and Claus, H. (2015) The potential of the yeast Debaryomyces hansenii H525 to degrade biogenic amines in food. Microorganisms, 3(4).

Bonnarme, P., Lapadatescu, C., Yvon, M. and Spinnler, H. E. (2001) L-methionine degradation potentialities of cheese-ripening microorganisms. Journal of Dairy Research, 68(4), pp. 663–674. https://doi.org/10.1017/S002202990100509X

Bourbon, A. I., Pinheiro, A. C., Cerqueira, M. A., Rocha, C. M. R., Avides, M. C., Quintas, M. A. C. and Vicente, A. A. (2011) Physico-chemical characterization of chitosan-based edible films incorporating bioactive compounds of different molecular weight. Journal of Food Engineering, 106, pp. 111–118.

Box, G. E. P. and Wilson, K. B. (1951) On the experimental attainment of optimum conditions. Journal of the Royal Statistical Society: Series B, 13(1), pp. 1–45.

Božanić, R. (2015) Povećanje tržišne vrijednosti sira. In: Matijević, B. (ed.) Sirarstvo u teoriji i praksi. Karlovac: Veleučilište u Karlovcu, pp. 189–197.

Calzada, J., del Olmo, A., Picon, A. and Nuñez, M. (2015b) Effect of high pressure processing on the lipolysis, volatile compounds, odour and colour of cheese made from unpasteurized milk. Food and Bioprocess Technology, 8, pp. 1076–1088. https://doi.org/10.1007/s11947-015-1473-4

Calzada, J., del Olmo, A., Picon, A., Gaya, P. and Nuñez, M. (2015a) Effect of high-pressure processing on the microbiology, proteolysis, biogenic amines and flavour of cheese made from unpasteurized milk. Food and Bioprocess Technology, 8, pp. 319–332. https://doi.org/10.1007/s11947-014-1406-7

Costabel, L. M., Bergamini, C., Vaudagna, S. R., Cuatrin, A. L., Audero, G. and Hynes, E. (2016) Effect of high-pressure treatment on hard cheese proteolysis. Journal of Dairy Science, 99, pp. 1–13. https://doi.org/10.3168/jds.2015-9907

Deepali Mohite and Waghmare, R. B. (2020) Encapsulation techniques for delivery of bioactive compounds in milk and dairy products: A review. Food Science and Technology, D. Y. Patil University, Navi Mumbai, India.

Espinosa-Pesqueira, D., Hernández-Herrero, M. M. and Roig-Sagués, A. X. (2018) High hydrostatic pressure as a tool to reduce formation of biogenic amines in artisanal Spanish cheeses. Foods, 7(9), 137. https://doi.org/10.3390/foods7090137

Fan, W. and Qian, M. C. (2006) Characterization of aroma compounds of Chinese "Wuliangye" and "Jiannanchun" liquors by aroma extract dilution analysis. Journal of Agricultural and Food Chemistry, 54(7), pp. 2695–2704. https://doi.org/10.1021/jf052635t

Fox, P. F. (1989) Proteolysis during cheese manufacture and ripening. Journal of Dairy Science, 72(6), pp. 1379–1400. https://doi.org/10.3168/jds.S0022-0302(89)79246-8

Gabriel'yants', M. A., Teplova, L. N., Karpova, T. I., Kozlova, R. A. and Makarova, G. F. (1980) Storage of hard rennet cheeses in cold stores with ozonization of air. Kholodil'naya Tekhnika, 5, pp. 35–37.

Gao, P., Su, Y., Zhang, W., Pang, X., Xie, N., Zhang, M., Lv, J. and Zhang, S. (2022) Chemical and flavor characteristics of enzyme-modified cheese made by two-stage processing. Gels, 8, 160. https://doi.org/10.3390/gels8030160

Gervilla, R., Ferragut, V. and Guamis, B. (2000) High pressure inactivation of microorganisms inoculated into ovine milk of different fat contents. Journal of Dairy Science, 83, pp. 674–682.

Gregurek, Lj. (2015) Proizvodnja sireva – teorija i praksa. Zagreb: PROBIOTIK d.o.o., ISBN: 978-953-58763-0-4

Guinee, T. P. and O'Brien, B. (2010) The quality of milk for cheese manufacture. In: Law, B. A. and Tamime, A. Y. (eds.) Technology of Cheesemaking. 2nd edn. Chichester: John Wiley & Sons, Ltd., Publication. ISBN 978-1-4051-8298-0

Güler, Z. (2005) Quantification of free fatty acids and flavor characteristics of Kasar cheeses. Journal of Food Lipids, 12(3), pp. 209–221. https://doi.org/10.1111/j.1745-4522.2005.00018.x

Havranek, J., Kalit, S., Antunac, N. and Samaržija, D. (2014) Sirarstvo. Zagreb: Hrvatska mljekarska udruga.

Hokmollahi, F. and Ehsani, M. R. (2017) High pressure processing and its application in cheese manufacturing: A review. Journal of Food Biosciences and Technology, 7(2), p. 57.

Jelovac, N., Perković, A., Pupovac, M., Trontel, A. and Slavica, A. (2011) Catabolism of amino acids in the cells of lactic acid bacteria. Croatian Journal of Food Technology, Biotechnology and Nutrition, 6(1–2), pp. 25–36.

Jin-Gab, K., Ahmed, E. Y. and Sandhya, D. (1999) Application of ozone for enhancing the microbiological safety and quality of foods: A review. Journal of Food Protection, 62(9), pp. 1071–1087. https://doi.org/10.4315/0362-028X-62.9.1071

Juric, M., Bertelsen, G., Mortensen, G. and Petersen, M. A. (2003) Light-induced colour and aroma changes in sliced, modified atmosphere packaged semi-hard cheeses. International Dairy Journal, 13(2–3), pp. 241–249. https://doi.org/10.1016/S0958-6946(02)00156-5

Kalit, S. (2015) Opće sirarstvo. In: Matijević, B. (ed.) Sirarstvo u teoriji i praksi. Karlovac: Veleučilište u Karlovcu, pp. 29–47. ISBN: 978-953-7343-79-8

Khoshgozaran, S., Hossein Azizi, M. and Bagheripoor-Fallah, N. (2012) Evaluating the effect of modified atmosphere packaging on cheese characteristics: A review. Dairy Science & Technology, 92(1), pp. 1–24. https://doi.org/10.1007/s13594-011-0044-3

Laili, I., Gunarso, S., Wathan, N., Sugijanto, N. E. and Indrayanto, G. (2017) Identification of Massoia lactone and its hydroxy-derivative from Kabatiella caulivora, an endophyte of the terrestrial plant Alyxia reinwardtii. Makara Journal of Science, 21(4), pp. 182–186. https://doi.org/10.7454/mss.v21i4.8840

Leclercq-Perlat, M. N., Sicard, M., Perrot, N., Trelea, I.-C., Picque, D. and Corrieu, G. (2015) Temperature and relative humidity influence the ripening descriptors of Camembert-type cheeses throughout ripening. Journal of Dairy Science, 98(2), pp. 1325–1335. https://doi.org/10.3168/jds.2014-8916

Linares, D. M., Cruz Martín, M., Ladero, V., Alvarez, M. A. and Fernández, M. (2011) Biogenic amines in dairy products. Critical Reviews in Food Science and Nutrition, 51, pp. 691–703.

Loizzo, M. R., Menichini, F., Picci, N., Puoci, F., Spizzirri, U. G. and Restuccia, D. (2013) Technological aspects and analytical determination of biogenic amines in cheese. Trends in Food Science & Technology, 30, pp. 38–55.

Lortal, S. and Chapot-Chartier, M. P. (2005) Role, mechanisms and control of lactic acid bacteria lysis in cheese. International Dairy Journal, 15(6–9), pp. 857–871. https://doi.org/10.1016/j.idairyj.2004.08.024

Mantle, P. G. (1987) Secondary metabolites of Penicillium and Acremonium. In: Peberdy, J. F. (ed.) Penicillium and Acremonium. Biotechnology Handbooks, vol. 1. Boston, MA: Springer, pp. 91–116. https://doi.org/10.1007/978-1-4899-1986-1_6

McHugh, T. (2015) Ozone processing of foods and beverages. Food Technology Magazine, 69(11).

McSweeney, P. L. H. and Sousa, M. (2000) Biochemical pathways for the production of flavour compounds in cheeses during ripening: A review. Le Lait, 80(3), pp. 293–324. https://doi.org/10.1051/lait:2000127

McSweeney, P. L. H. (2004) Biochemistry of cheese ripening. International Journal of Dairy Technology, 57(2–3), pp. 127–144. https://doi.org/10.1111/j.1471-0307.2004.00147.x

McSweeney, P. L. H. (2007) Cheese manufacture and ripening and their influence on cheese flavour. In: Weimer, B. C. (ed.) Improving the flavour of cheese. Cambridge: Woodhead Publishing, pp. 1–20.

Mikulec, N., Habuš, I., Antunac, N., Vitale, Lj. and Havranek, J. (2010) Formiranje arome sira. Mljekarstvo, 60(4), pp. 219–227.

Mohammadi, R., Mahmoudzade, M., Atefi, M., Khosravi-Darani, K. and Mozafari, M. R. (2014) Applications of nanoliposomes in cheese technology. International Journal of Dairy Technology, 68(1), pp. 11–23. https://doi.org/10.1111/1471-0307.12174

Nair, M. K. M., Joy, J., Vasudevan, P., Hinckley, L., Hoagland, T. A. and Venkitanarayanan, K. S. (2005) Antibacterial effect of caprylic acid and monocaprylin on major bacterial mastitis pathogens. Journal of Dairy Science, 88(10), pp. 3488–3495. https://doi.org/10.3168/jds.S0022-0302(05)73033-2

Nájera, A. I., Nieto, S., Barron, L. J. R. and Albisu, M. A. (2021) A review of the preservation of hard and semi-hard cheeses: Quality and safety. International Journal of Environmental Research and Public Health, 18(18), 9789. https://doi.org/10.3390/ijerph18189789

Nuñez, M., Calzada, J. and del Olmo, A. (2020) High pressure processing of cheese: Lights, shadows and prospects. International Dairy Journal, 100, 104558. https://doi.org/10.1016/j.idairyj.2019.104558

O'Reilly, C. E., Kelly, A. L., Oliveira, J. C., Murphy, P. M., Auty, M. A. E. and Beresford, T. P. (2003) Effect of varying high-pressure treatment conditions on acceleration of ripening of Cheddar cheese. Innovative Food Science & Emerging Technologies, 4(3), pp. 277–284. https://doi.org/10.1016/S1466-8564(03)00037-7

O'Reilly, C. E., Kelly, A. L., Murphy, P. M. and Beresford, T. P. (2001) High pressure treatment: Applications in cheese manufacture and ripening. Trends in Food Science & Technology, 12(2), pp. 51–59. https://doi.org/10.1016/S0924-2244(01)00060-7

Oštarić, F., Antunac, N., Cubric-Curik, V., Curik, I., Jurić, S., Kazazić, S., Kiš, M., Vinceković, M., Zdolec, N. and Špoljarić, J. (2022) Challenging sustainable and innovative technologies in cheese production: A review. Processes, 10, 529. https://doi.org/10.3390/pr10030529

Ozturk, M., Govindasamy-Lucey, S., Jaeggi, J. J., Johnson, M. E. and Lucey, J. A. (2013) The influence of high hydrostatic pressure on regular, reduced, low and no salt added Cheddar cheese. International Dairy Journal, 33, pp. 175–183.

Pandiselvam, R., Singh, A., Agriopoulou, S., Sachadyn-Król, M., Aslam, R., Gonçalves Lima, C. M., Khanashyam, A. C., Kothakota, A., Atakan, O., Kumar, M., Mathanghi, S. K. and Khaneghah, A. M. (2022) A comprehensive review of impacts of ozone treatment on textural properties in different food products. Trends in Food Science & Technology, 127, pp. 74–86. https://doi.org/10.1016/j.tifs.2022.06.008

Panfil-Kuncewicz, H., Kuncewicz, A. and Lis, A. (2014) The effect of gas composition in modified atmosphere packaging on the shelf life of portioned ripened cheese. In: Chochół, A. and Szakiel, J. (eds.) Commodity Science in Research and Practice – Achievements and Challenges of Commodity Science in the Age of Globalization. Cracow: Polish Society of Commodity Science, pp. 193–200. ISBN: 978-83-938909-0-3.

Papademas, P. and Bintsis, T. (2017) Traditional wooden equipment used for cheesemaking and their effect on quality. In: Global Cheesemaking Technology. Chichester: John Wiley & Sons, pp. 157–172. https://doi.org/10.1002/9781119046165.ch0g

Rodriguez, E., Arques, J. L., Nunez, M., Gaya, P. and Medina, M. (2005) Combined effect of high-pressure treatments and bacteriocin-producing lactic acid bacteria on inactivation of Escherichia coli O157:H7 in raw-milk cheese. Applied and Environmental Microbiology, 71(7), pp. 3399–3404. https://doi.org/10.1128/AEM.71.7.3399-3404.2005

Shiler, G. G., Eliseeva, N. N., Volodin, V. I., Chebotarev, L. N. and Matevosyan, L. S. (1983) Method of ozonizing rooms for ripening and storing cheeses. USSR patent SU1022688A.

Skeie, S. (2010) Milk quality requirements for cheesemaking. In: Griffiths, M. W. (ed.) Improving the Safety and Quality of Milk. Cambridge: Woodhead Publishing, pp. 433–453. https://doi.org/10.1533/9781845699437.3.432

Sousa, M. J., Ardö, Y. and McSweeney, P. L. H. (2001) Advances in the study of proteolysis during cheese ripening. International Dairy Journal, 11(4–7), pp. 327–345. https://doi.org/10.1016/S0958-6946(01)00062-0

Styles, M. F., Hoover, D. G. and Farkas, D. F. (1991) Response of Listeria monocytogenes and Vibrio parahaemolyticus to high hydrostatic pressure. Journal of Food Science, 56, pp. 1404–1407.

Šutić, M., Vilušić, S., Obradović, D., Davitkov, A. and Marković, D. (1980) Primena polivalentne kulture za zrenje sireva u plastičnim folijama. Mljekarstvo, 30(5).

Terpou, A., Bosnea, L., Kanellaki, M., Plessas, S., Bekatorou, A., Bezirtzoglou, E. and Koutinas, A. A. (2018) Growth capacity of a novel potential probiotic Lactobacillus paracasei K5 strain incorporated in industrial white brined cheese as an adjunct culture. Journal of Food Science. https://doi.org/10.1111/1750-3841.14079

Thierry, A., Collins, Y. F., Abeijón Mukdsi, M. C., McSweeney, P. L. H., Wilkinson, M. G. and Spinnler, H. E. (2017) Lipolysis and metabolism of fatty acids in cheese. In: Cheese: Chemistry, Physics and Microbiology. 4th edn. London: Academic Press, pp. 423–444. https://doi.org/10.1016/B978-0-12-417012-4.00017-X

Tian, H., Sun, X., Yu, H., Ai, L. and Chen, C. (2020) Characterization of the key aroma compounds in Yunnan goat milk cake using a sensory-directed flavor analysis. Journal of Food Science, 85, pp. 3981–3997. https://doi.org/10.1111/1750-3841.15490

Tian, H., Xu, X., Chen, C. and Yu, H. (2019) Flavoromics approach to identifying the key aroma compounds in traditional Chinese milk fan. Journal of Dairy Science, 102(11), pp. 9639–9650. https://doi.org/10.3168/jds.2019-16796

Tidona, F., Zago, M., Carminati, D. and Giraffa, G. (2022) The reduction of salt in different cheese categories: Recent advances and future challenges. Frontiers in Nutrition, 9, 859694. https://doi.org/10.3389/fnut.2022.859694

Timilsena, Y., Haque, M. and Adhikari, B. (2020) Encapsulation in the food industry: A brief historical overview to recent developments. Food and Nutrition Sciences, 11, pp. 481–508. https://doi.org/10.4236/fns.2020.116035

Tudor Kalit, M., Lojbl, T., Rako, A., Gun, I. and Kalit, S. (2020) Biochemical changes during ripening of cheeses in an animal skin. Mljekarstvo, 70(4), pp. 225–241.

Varga, L. and Szigeti, J. (2016) Use of ozone in the dairy industry: A review. International Journal of Dairy Technology, 69(2), pp. 157–168. https://doi.org/10.1111/1471-0307.12302

Vesonder, R. F., Stodola, F. H. and Rohwedder, W. K. (1972) Formation of the δ-lactone of 3,5-dihydroxydecanoic acid by the fungus Cephalosporium recifei. Canadian Journal of Biochemistry, 50(4), pp. 363–365. https://doi.org/10.1139/o72-048

Zheng, X., Shi, X. and Wang, B. (2021) A review on the general cheese processing technology, flavor biochemical pathways and the influence of yeasts in cheese. Frontiers in Microbiology, 12. https://doi.org/10.3389/fmicb.2021.703284