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Effect of milk thistle (Silybum marianum) seed flour on chemical, microbial and sensory properties of soy protein-plant based meat.

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Dedication

I dedicate this thesis...

To my parents Ali and Majeda. Without their endless love and encouragement, I would never have been able to complete my graduate studies. I love you both and I appreciate everything that you have done for me.

To my beloved family big and small who supported and encouraged me through all the stages.

To everyone who helped me complete this study.

With respect and love.

declaration

I, Shifaa Abu atwan, hereby declare that I am the author of this thesis. All the work described in this thesis is my own except where stated in the text. The work presented here has not been accepted in any previous application for a higher degree. All the source of information have been consulted by myself and are acknowledged by means of reference.

Signed:

Shifaa Ali Abu Atwan

Date: 27/8/2023

Acknowledgment

It is with great pleasure that I write this section of my thesis. In my heart and mind, it is the most important part of this thesis. I get to say “thank you” to all the wonderful people who have made it possible for me to go through this program. My deepest gratitude is to Allah my maker. I am extremely thankful to Allah for blessing me with life, a good health, the intellect and resources to be able complete this program. In my most difficult moments, the word of Allah always served to up life my sprit, and give me a sense of purpose.

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I must thank my parents Ali and Majeda, my sisters and brothers, nephews and nieces who all gave me endless encouragement, inspiration, support and advice.

Abstract

This study was conducted to investigate the effect of milk thistle seed flour on microbial, chemical and sensory properties of soy plant-based meat analogue. Three formulas for the soy protein plant-based meats were created, each of which had a different proportion of milk thistle seed flour (MTSF) and wheat flour (WT): Sample 1 contained 28% MTSF, Sample 2 contained 14% MTSF and 14% WF, and Sample 3 contained 28% WF. Microbial results illustrate that sample containing 28% MTSF exhibited high total plate counts (8±1 CFU/g) compared to other samples at the initial period, but after six months of storage, the same sample exhibited low counts (1±1 CFU/g). The highest yeast and mold count was observed in sample 3, which contains 28% MTSF (3±1.4 CFU/g) in the sixth month of storage. During the six months of freezing storage, coliform counts in all samples tested positive and varied from 2 to 7.67 CFU/g. Meat analogue samples that contain 14% MTSF have a significantly high ($P< 0.05$) coliform count at the initial period. Overall, all counts decreased significantly during the storage period. The chemical composition results illustrate that soy plant-based meat analogues containing MTSF had lower moisture and carbohydrate content, whereas they had higher protein, fat ash, and crude fiber than soy plant-based meats containing WF. During a frozen storage period, there is an unnoticeable decrease in the protein, fat, carbohydrate, fiber, and ash content of all meat analogue samples. Additionally, samples that contain 28% MTSF show an increase in moisture content of 0.43% after six months of storage. While moisture content decreased during 6 months frozen storage in both samples 2 and 3. The meat analogue samples with 0% MTSF after six months of storage had the highest peroxide value (PV) recorded. PV values of this sample was increased from 0.65 mEq/g at initial time to 1.70 mEq/g at six month of storage period. On other hand, the highest thiobarbituric acid reactive substances (TBARs) value was reported in meat analogue samples containing 0% MTSF during storage. TBARs values of this sample increased from 0.21mg /kg at initial time to 0.27 mg/kg at sixth month of storage period. PV and TBARS levels increased noticeably with storage time. Overall, panelists' in the sensory analysis presented that soy plant based meat analogue containing 0% and 14% of MTSF had better sensory properties than soy plant based meat analogue containing 28% of MTSF.

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Chapter 1

Introduction

1.1. Meat Analogues:

The projected global population of 9.7 billion by 2050, as predicted by the United Nations (2019), is expected to result in an increased need for protein. However, the consumption of meat and dairy products, which serve as the primary protein source for many people in developed nations, is on the rise worldwide. Unfortunately, the production of these animal-based products requires significant resources such as arable land, pastures, livestock feed, and water (Godfray et al., 2018). Consequently, concerns arise regarding the feasibility of providing affordable food for the growing population without causing environmental harm (Kyriakopoulou et al., 2019). In this context, plant-based foods offer a viable solution to meet the global food demand sustainably and in a nutritionally balanced manner.

In their work, Shurtleff and Aoyagi (2014) presented a comprehensive timeline that documented numerous historical events related to meat alternatives worldwide, covering the period from 965 to 2014. According to their research, the earliest known mention of a soy-based tofu product dates back to 965 in China. Over time, there were several advancements and references to plant-based meat analogues throughout history. However, it wasn't until the mid to late-1900s that significant progress in food technology began to take place in this field. Notably, many of the modern techniques utilized in the production of meat analogues, such as biopolymer spinning and extrusion, were initially patented in 1947 and 1954, respectively.

Throughout history, the composition of primary protein ingredients in meat analogues has undergone changes. Historical records indicate the use of various ingredients, such as tofu (a soy product) dating back to 965, wheat protein dating back to 1301, yuba (a soy product) dating back to 1587, tempeh (a soy product) dating back to 1815, and combinations of nuts, cereals, and legumes dating back to 1895 (Shurtleff & Aoyagi, 2014). These references highlight the diverse range of protein sources that have been utilized in the development of meat analogues over the centuries.

The emergence of the new generation of meat analogs, such as Beyond Meat, Impossible Burgers, and Garden, exemplifies successful innovations utilizing these protein sources. The plant-based meat market has experienced significant growth, with projections indicating a rise from $4.6 billion in 2018 to an estimated $85 billion by 2030. As a significant milestone, it is anticipated to reach $30.9 billion by 2026 (Sha & Xiong, 2020). These figures demonstrate the substantial market potential and consumer interest in plant-based meat alternatives in the coming years.

The global demand for plant-based meat substitutes is on the rise due to various factors, including concerns related to animal welfare, sustainability, and health benefits (Hargreaves et al., 2020; Raphaely et al., 2013). Meat has traditionally been a significant component of the dietary habits in Western populations, as it serves as a source of essential nutrients like proteins, fats, minerals such as iron and zinc, as well as vitamins A and B12 (Demartini et al., 2022).

Although global data regarding the number of consumers following meat-free diets is not available, statistics show significant numbers in different regions. In Asia, approximately 19% of the population follows meat-free diets, followed by Africa with 16%, South and Central America with 8%, and North America with 6% (Statista, 2016). Additionally, there has been a continuous increase in the number of individuals adopting diets that eliminate or reduce meat and meat products in recent years (Estruch et al., 2021).

The social aspect of food and its influence on human interactions is another important factor driving the growing demand for plant-based meat substitutes (Demartini et al., 2022; Asher & Peters, 2021). Therefore, there is a need for alternative food products that can replace meat and cater to the preferences and dietary choices of the global population.

Meat analogues are plant-based protein products that offer several advantages, including a high content of essential amino acids, low saturated fat, and no cholesterol (Berk, 1992; Riaz, 2001). Consumption of a plant protein-based diet has been linked to benefits such as weight reduction, lower cholesterol levels, and decreased blood pressure, leading to a reduced risk of stroke, heart disease, and certain types of cancer (Tuso et al., 2015). Consequently, the substitution of meat protein with plant protein has garnered attention from both academic and industrial researchers (Apostolidis & Mcleay, 2016; Asgar et al., 2010; Hoek et al., 2011).

Meat analogues are designed to resemble meat in terms of their aesthetic properties, including structure, texture, flavor, color, and appearance (Asgar et al., 2010; Strahm, 2006). They are primarily composed of texturized vegetable proteins, which create a fibrous matrix that mimics the fibrillar structure of meat muscle (Caporgno et al., 2020). Various techniques for structuring vegetable proteins have been extensively explored in recent years (Kyriakopoulou et al., 2019; Dekkers et al., 2018; Zhang et al., 2019).

The conventional process for developing meat analogues typically involves two primary steps: mixture preparation and chunk formation (Malav et al., 2015; Orcutt et al., 2006). In the mixture preparation stage, the proteins, fats, salts, and other ingredients are blended, chopped, and mixed to create a matrix of proteins that encapsulates the fat and non-soluble components. This blending of ingredients can take place either prior to extrusion or within the extruder itself. During this process, water is added to achieve a final moisture content of around 60% (Chen et al., 2010; Lin & Hsieh, 2002). Once water is injected into the extruder, the ingredients are heated to temperatures exceeding 150 °C and then forced through a cooling die (Cheftel et al., 1992). The heat and shear forces during extrusion cause protein denaturation, leading to the formation of new chemical bonds between adjacent protein molecules and the creation of a fibrous, meat-like structure (Cheftel et al., 1992).

The most commonly utilized technology for producing meat analogues is high-moisture extrusion cooking using a twin-screw extruder. This method generates relatively large pieces of texturized protein that resemble muscle meat, making it suitable for developing chunk-type meat analogues (Osen et al., 2014; Sha & Xiong, 2020).

Currently, the meat analogues available on the market primarily include burgers and patties (Bohrer, 2019). However, a low-moisture extrusion cooking method could be more advantageous as it produces texturized protein in the form of small pellets with a fibrous texture. These pellets can be easily rehydrated and mixed with the necessary ingredients to produce finished meat analogues (Angelis et al., 2020).

The raw materials commonly used for texturization in meat analogues are protein isolates derived from soy (Tehrani et al., 2017) and pea (Osen et al., 2014). However, there is growing interest in exploring other protein sources such as lupin (Palanisamy et al., 2019), hemp (Zahri et al., 2020), mung bean, and wheat gluten (Samard & Ryu, 2019).

Typically, plant-based meat substitutes are composed of a combination of legumes and cereals, employing various technologies depending on the desired characteristics of the final product. Food additives may also be incorporated to enhance flavor, texture, and appearance (Onwezen et al., 2021).

Legumes are considered significant plant sources rich in proteins and valuable nutritional compounds. When combined with cereals, they can be prepared to provide a considerable amount of high-quality protein, ranging from 17% to 38%. In comparison, the protein content in grains of legumes is two to three times higher than that found in cereals, which typically contain 10% to 15% protein (Boye et al., 2010). Legumes can be utilized as abundant sources of protein in the production of plant protein byproducts in the form of flour (50%-65% protein), concentrates (65%-90% protein), and protein isolates (over 90%).

Soy protein, in particular, can serve as an ideal basis for texturized vegetable protein, allowing for the creation of soy-based meat alternatives. Soy products are excellent substitutes for animal-based products as they provide consumers with essential amino acids and offer a comparable protein quality to animal-based products (Zhang et al., 2021; Jooyandeh, 2011). Soy is low in saturated fat and rich in vitamins and minerals, further enhancing its nutritional value. Texturized vegetable protein also exhibits year-long shelf stability in its dry form and has the capacity for rehydration without compromising its structure, shape, and chewy texture (Harper, 1981).

Soy proteins are rich in lysine, making them valuable supplements for cereals, which tend to be low in this essential amino acid. However, methionine is the first limiting amino acid in soy proteins, and this limitation should be considered when adding soy proteins for nutritional purposes rather than solely for functionality (Wolf, 1970; Morita et al., 1997).

In recent years, soy-based products have gained increasing attention due to their potential in improving health conditions, particularly cardiovascular diseases and other chronic diseases (Jooyandeh, 2011). Soy proteins have a digestibility similar to that of animal-based products and may even have better digestibility than other legume proteins or cereals (Choudhury et al., 2020). Additionally, soy has a higher protein content and lower carbohydrate content compared to other legumes. Protein quality is often cited as a key reason for the research interest in soy products (Rizzo & Baroni, 2018). Furthermore, soy is considered environmentally friendly, with a relatively good ecological footprint. Soy cultivation also has the ability to bind nitrogen, contributing to its environmental benefits (Soja Netzwerk Schweiz, 2019).

The quantity and quality of soy protein play important roles in determining the chemical and physical characteristics of meat analogues. Increasing the protein content in meat analogues can result in higher shearing forces and finer texture in the extrudate (Maurice & Stanley, 1978). Soy carbohydrates also contribute to the overall structure of soy protein extrudates (Sheard et al., 1984).

Cereal proteins, which can be derived from various sources such as wheat, rice, barley, and oats, are commonly used in meat analogues (Malav et al., 2015). Wheat has been historically widely used in meat analogue products, but modern products often include rice, barley, and oat ingredients. Cereal ingredients are generally higher in carbohydrate content and lower in protein content compared to soy. However, when considering the protein content of cereal ingredients alone, their protein digestibility scores are typically lower compared to other protein sources due to suboptimal amino acid profiles and lower protein digestibility (Joye, 2019).

Cereal proteins generally have lower values for many amino acids compared to other protein sources, with lysine being the first limiting amino acid in cereal proteins (Vk & Satishk, 2016; Joye, 2019; Mota et al., 2016).

The digestibility of cereal proteins can be influenced by both internal factors, such as structural features, protein folding/crosslinking, and amino acid sequence, and external factors, such as anti-nutritional factors like protease inhibitors, tannins, and phytates (Joye, 2019). From a functional standpoint, cereal proteins are valuable to manufacturers of meat analogues due to their structural properties. Most cereal proteins exhibit a viscoelastic structural network, which contributes to successful binding and provides the desired consistency and fibrous-like texture in meat analogues (Kumar et al., 2016; Kyriakopoulou et al., 2019; Malav et al., 2015).

Protein ingredients play a crucial role in defining the identity and differentiation of meat analogues. Proteins have various structure-function relationships, including hydration and solubility, interfacial properties (emulsification and foaming), flavor binding, viscosity, gelation, texturization, and dough formation (Meade et al., 2005; Damodaran & Parkin, 2017). Additionally, during processing, proteins undergo physical, chemical, and nutritional changes that depend on the protein source (Meade et al., 2005; Damodaran & Parkin, 2017; Friedman, 1996; Foegeding & Davis, 2011).

Animal-derived products, such as meat, are considered complete sources of protein as they provide all the essential amino acids in adequate proportions and have good digestibility (Bohrer, 2019). In contrast, plant-based protein sources may lack or be limiting in one or more essential amino acids and their digestibility can be compromised due to various factors (Bohrer, 2017; Huang et al., 2018; Young & Pellet, 1994).

Meat itself does not naturally contain carbohydrates, unless additional carbohydrate ingredients are added during processing, which is common in processed meat products, especially emulsified and formed products (Tarte, 2009). On the other hand, meat analogue products often contain carbohydrates. These carbohydrates can come from various ingredients and serve different purposes in the manufacturing process. Carbohydrate ingredients can include starches or flours used to improve texture and consistency, as well as binding ingredients or gums like methylcellulose, acacia gum, xanthan gum, carrageenan, and others, which enhance product stability and form (Kumar et al., 2016; Kyriakopoulou et al., 2019; Tarte, 2009). Functionally, these ingredients facilitate interaction between the protein, lipid, and water components of the food system, promoting stability and structure formation. From a nutritional standpoint, carbohydrates can be beneficial as dietary fiber or have potential health risks when consumed in the form of refined starches or sugars (Topping, 2007; Viuda-Martos et al., 2010).

In both meat analogue products and processed meat products, a combination of dietary fiber, starches, and sugars is commonly included in the formulation (Kumar et al., 2016; Kyriakopoulou et al., 2019; Tarte, 2009). It can be challenging to determine the specific health advantages of the carbohydrate ingredients added to meat analogue products. One ingredient often used is methylcellulose, which is a modified cellulose dietary fiber and an effective binder when included in appropriate quantities in food products (Schuh et al., 2013). From a nutritional perspective, methylcellulose forms a viscous solution in the gastrointestinal tract and has shown similar effects on glucose metabolism as other sources of dietary fiber (Jenkis et al., 1978; Topping et al., 1988; Maki et al., 2008).

Traditionally, meat analogues have been low in lipid content (Kumar et al., 2016; Kyriakopoulou et al., 2019). However, modern meat analogue products now often contain higher lipid content, comparable to traditional meat products. Various lipid ingredients, such as canola (rapeseed) oil, coconut oil, sunflower oil, corn oil, sesame oil, and cocoa butter, among others, are used in the formulation of meat analogues (Kyriakopoulou et al., 2019). Fats and oils play a role in contributing to the juiciness, tenderness, mouthfeel, and flavor release of the product. However, it is important to consider the effects of fats and oils during processing and preparation to avoid excessive lubrication and stickiness (Kyriakopoulou et al., 2019).

From a nutrition standpoint, the healthiness of dietary fats and oils in the human diet has been a subject of debate. Generally, nutritionists, dieticians, and organizations like the American Heart Association recommend dietary patterns that limit the consumption of saturated fats and trans fats while promoting the consumption of unsaturated fats (Karuss et al., 1996; Appel et al., 2006; Sacks et al., 2017).

The fatty acid composition of plant-based fats and oils differs substantially from animal-derived fats, with varying proportions of short-chain, intermediate-chain, and long-chain fatty acids. Numerous research studies have examined the fatty acid composition of different plant-based fats and oils to further evaluate their nutritional characteristics (Chowdhury et al., 2007; Zambiazi et al., 2017; Kostik et al., 2013).

The fatty acid composition of sunflower oil can vary depending on manufacturing specifications. As an example, a brand of sunflower oil found in the USDA Nutrient Database (NBD ID: 45212857) is reported to contain approximately 6.67 g of saturated fat, 53.33 g of monounsaturated fat, 26.67 g of polyunsaturated fat, and 0.00 g of trans fat per 100 g of the product (U.S. Department of Agriculture, 2019). The major fatty acids in the composition of sunflower oil include long-chain fatty acids such as palmitic acid (C16:0), stearic acid (C18:0), oleic acid (C18:1), and linoleic acid (C18:2).

The color of meat products is indeed an important attribute for consumer preferences. The characteristic color of uncooked meat products, such as bright cherry red for beef, reddish pink for pork, and bluish-white to yellow for poultry, is primarily due to the presence of myoglobin, a protein responsible for meat color (Mancini & Hunt, 2015). During cooking, the color of meat products changes as myoglobin undergoes chemical changes.

Similar considerations apply to meat analogue products, which aim to replicate the color attributes of meat before, during, and after cooking (Kyriakopoulou et al., 2019). The specific ingredients used to achieve the desired colors in modern meat analogue products may vary. In some cases, naturally occurring colorants are used, such as beet juice extract in the Beyond Burger and tomato paste in the MorningStar Farms burger. Another approach involves using sarcoplasmic proteins that have a similar chemical structure to myoglobin, such as reduced iron compounds in the Gardein meatless meatballs or soy leghemoglobin in the Impossible Burger. Soy leghemoglobin is a soy-derived compound that shares chemical and structural features with hemoglobin and myoglobin (Fraser et al., 2018; Robinson, 2019).

The safety risks associated with soy leghemoglobin are still being studied, but current research suggests no significant toxicological concerns with its use as an ingredient (Fraser et al., 2018; Robinson, 2019).

1.2 Milk thistle:

Milk thistle (*Silybum marianum*) is a plant belonging to the Asteraceae family. It is an annual or biennial plant native to the Mediterranean area and some parts of the United States, but it has now spread to other warm and dry regions (Hadolin et al., 2001). The Asteraceae family is one of the largest plant families, with over 1600 genera and approximately 23,000 species, mostly found in temperate regions (Jeffrey, 2007).

Milk thistle has a long history of use in traditional medicine. The herbalist John Gerard, in his book "Generall Historie of Plantes," described milk thistle as the best remedy for melancholy diseases. It has been recognized for its potential medicinal properties and has been used for over 2000 years to treat liver and biliary disorders, including cirrhosis, chronic hepatitis, and exposure to environmental contaminants. The seeds of milk thistle, known as milk thistle seeds (MTS), are particularly valued for their therapeutic properties. They are considered one of the most common botanical supplements used worldwide for therapeutic purposes. These seeds are known to contain bioactive compounds, including flavonolignans (silymarin) and other flavonoids, which are believed to contribute to their medicinal effects (Albassam et al., 2017).

Studies have shown that milk thistle seeds can have hepatoprotective (liver-protecting) effects, antioxidant properties, and potential anti-inflammatory and anticancer activities. However, further research is still needed to fully understand the mechanisms and potential benefits of milk thistle in various health conditions (Albassam et al., 2017).

Figure 1.1

Milk thistle (*Silybum marianum*) is considered an important medicinal crop in Europe, particularly in Poland, where it is a significant producer of MTS and medicines derived from them. In recent years, the importance of MTS has also grown in North America (Karkanis et al., 2011).

The main active component of milk thistle seeds is silymarin, which is a complex mixture of compounds. Silymarin consists of approximately 70-80% flavonolignans, including silybin, silydianin, silychristin, isosilybin, isosilychristin, dehydrosilybin, and silandrin. It also contains flavonoids such as taxifolin and quercetin, as well as other polyphenol-like molecules that have not been precisely characterized chemically (Pardhan & Girish, 2006).

Silymarin is found in various parts of the milk thistle plant, including the seeds, fruit, and leaves, but the highest concentration is typically found in the seeds (Hobbs, 2008). The content of silymarin in the fruit may vary depending on the milk thistle variety and the geographic and climatic conditions in which it grows (Ghahreman, 1999).

MTS have been found to contain approximately 26.05% oil, 4.48% moisture, 1.93% ash, 5.48% crude fiber, 87.2% carbohydrates, and 23% total proteins (Khan et al., 2007). Additionally, milk thistle fruits are known for their high iron content, comparable to that of dry soybean seeds. Studies conducted on rats have shown that the iron content in MTS is readily assimilated and can lead to an increase in hemoglobin content in their blood. This suggests that similar assimilation of iron could occur in humans (Kunachowicz et al., 2000; Jadayil et al., 1999).

Thistle seeds, rich in silymarin, have been found to exhibit antioxidant activity, anti-inflammatory properties, and potential benefits against carcinogenesis, atherosclerosis, hypertension, diabetes, and obesity (Zhang et al., 2020; Kazazis et al., 2014; Fanoudi et al., 2016; Bhattacharya, 2020).

The antioxidant activity of silymarin plays a crucial role in inhibiting the formation of free radicals and lipid peroxidation, which can cause liver toxicity. Silymarin has been shown to protect hepatocyte membranes and limit cell lysis. It can also chelate iron and copper ions and influence the biosynthesis of molecules that protect the body from stress (Abenavvoli et al., 2018; Bhattacharya, 2020).

In vitro studies comparing silymarin to standard antioxidants such as butylated hydroxyanisole, butylated hydroxytoluene, α-tocopherol, and trolox have demonstrated its effective antioxidant and radical scavenging activity. This suggests that silymarin could be utilized to minimize or prevent lipid oxidation in pharmaceutical and food products, thereby preserving their nutritional quality and extending their shelf life (Köksal et al., 2009).

Furthermore, the potential inclusion of pure silymarin in human food, especially in areas affected by environmental pollution, has been suggested (Bongiovanni et al., 2007). This highlights the potential use of silymarin as a rich source of antioxidants and food preservatives.

Silymarin, the complex of substances found in milk thistle seeds, has been found to possess immunostimulatory effects (Thyagarajan et al., 2002). Additionally, it has demonstrated excellent antibacterial activity against certain microorganisms (de Oliveira et al., 2015) and antiviral activity against certain viruses (Wagoner et al., 2010).

MTS also contain betaine and trimethyl glycine (Ramasamy & Agarwal, 2008). The seed oil, specifically the ether extract, is a rich source of vitamin E and polyunsaturated fatty acids, particularly linoleic and oleic acids, which contribute to the hepatoprotective effects of milk thistle.

The volatile components of milk thistle seed oil have been analyzed, and γ-cadinene and α-pinene were found to be the predominant compounds (Mhamdi et al., 2016).

The protein content in the milk thistle seed kernel, primarily albumin, is highly nutritious in terms of essential amino acids and exhibits excellent processing properties. It has better solubility, foaming capacity, foam stability, and emulsification capacity compared to soy protein isolate (Chen et al., 1998; Zhu et al., 2011). Thus, the protein from milk thistle seed kernel has the potential to be used as a valuable source of protein nutrition.

The milk thistle protein, known as *Silybum marianum* protein (SMP), possesses an excellent balance of essential amino acids, with relatively high levels of glutamic acid, arginine, leucine, and valine. The nutritional quality parameters based on its amino acid composition indicate that SMP has good nutritional quality. In vitro digestion models have shown that SMP is easily digested by pepsin and trypsin. These functional properties make SMP suitable for potential applications as functional food ingredients, such as protein extenders in adhesives or in formulating acid foods, meat and milk analog products, and protein-rich beverages (Zhu et al., 2013).

Dietary fiber can be categorized into soluble and insoluble fractions. Soluble fiber, including oligosaccharides and indigestible polysaccharides, increases viscosity in the intestines, slows down digestion, and reduces the absorption of glucose and sterols. Oat bran, barley, beans, lentils, peas, fruits, and vegetables are valuable sources of soluble fiber (He et al., 2022; Gidley & Yakubov, 2019; Fuentes-Zaragoza et al., 2010; Prosky, 2000; Choct, 2015; Tejeda & Kim, 2021; Kim & Je, 2016; Xu et al., 2022). On the other hand, insoluble fiber mainly consists of cellulose, hemicelluloses, and lignin, and can be found in wheat bran, whole grains, nuts, and seeds. Consuming insoluble fiber has been associated with benefits such as weight management, improved insulin sensitivity, reduced blood pressure, and enhanced immune function (Xu et al., 2022; Makki et al., 2018; Weickert & Pfeiffer, 2018).

MTS can be consumed raw or prepared as tea (Bhattacharya, 2011). Milk thistle tea, rich in flavonoid silymarin, exhibits antioxidant properties that promote liver health, lower LDL cholesterol levels, and reduce damage to nucleic acids, lipid membranes, and proteins by neutralizing reactive oxygen species (ROS). The antioxidant capacity of milk thistle tea has been found to be superior to that of black tea.

A study by Apostol et al. (2017) investigated the use of partially defatted milk thistle seed flour (MTSF) in bakery products. It was found that replacing 5%, 10%, or even 15% of wheat flour with MTSF did not negatively affect the rheological parameters of the dough, indicating that MTSF can be incorporated into bakery products without compromising their quality. Furthermore, milk thistle seed proteins have a high content of essential amino acids, such as lysine, isoleucine, leucine, valine, and threonine, which are not present in wheat flour.

The phenolic content and antioxidant activities of milk thistle flour have been found to be higher than similar flours from pumpkin, parsley, mullein, and cardamom. This suggests that incorporating milk thistle seed flour into the human diet is recommended (Parry et al., 2008). Research on the biological activities of silymarin and silibinin, the compounds found in milk thistle, has increased due to their various pharmacological effects and the reported safety of their use with few adverse effects (Křen & Walterová, 2005; Jacobs et al., 2002). Silibinin, in particular, has demonstrated antioxidant, anti-inflammatory, antitumor, and anti-arthritic properties in both in vivo and in vitro studies (Křen & Walterová, 2005). It has also shown antibacterial activity against Gram-positive bacteria, such as *Bacillus subtilis* and *Staphylococcus epidermidis* (Dong et al., 2003).

Phenolic compounds, including flavonoids and lignans, have been investigated for their therapeutic potential as antimicrobial agents, with these compounds believed to be responsible for their antimicrobial activity (Michelin, 2005; Zuanazzi & Montanha, 2004). Studies have shown that silymarin exhibits antibacterial and antiadherent/antibiofilm activity against certain bacterial strains, indicating its potential benefits as a dietary supplement or drug (Evren & Yurtcu, 2015).

Phenolic compounds are believed to form complexes with extracellular soluble proteins that bind to the bacterial cell wall, contributing to their antibacterial action (Tsuchiya et al., 1996). Flavonoids have been shown to inhibit DNA topoisomerase activity by forming complexes that alter enzyme binding, suggesting a mechanism for their antibacterial effect (Wang, 2010). Additionally, the presence of hydroxyl phenolic groups in these compounds may interfere with bacterial synthetic processes by inhibiting enzymes, further contributing to their antibacterial activity (Avila et al., 2008; Li et al., 2012).

Milk thistle contains phenolic compounds, particularly silymarin and silibinin, which have been associated with a range of beneficial biological activities, including antioxidant, anti-inflammatory, antitumor, and antibacterial effects. These compounds have shown potential as antimicrobial agents and may interfere with bacterial cell wall binding and enzyme activity, contributing to their antibacterial properties.

In summary, MTS offer not only fiber but also antioxidant compounds and nutritional components that can be beneficial for liver health, cholesterol management, and the development of functional food products**.**

1.3. Study objectives:

This study aimed to investigate if the milk thistle seed flour (MTSF) can be used as flour add to meat analogous (soy protein-plant based meat). In order to achieve the objective of this research study chemical, microbial and sensorial properties of milk thistle seed flour on soy protein-plant based meat have been evaluated. In addition, to find out if milk thistle has antioxidant effect on soy protein- plant based meat.

In addition, Q comparison study between microbial and sensorial attributes of milk thistle seed flour (MTSF) and wheat flour on soy protein (SP) –plant based meat have been carried out.

Besides, to investigate how freezing affects the chemical and microbiological characteristics of soy protein-plant based meat during preservation. As well, to investigate the effect of milk thistle seed flour on the quality of soy protein-plant based meat during a storage period that extends up to six months at a temperature of (-18 ^{o}C) have been carried out.

Chapter 2

Literature review

Aziz et al. (2020) conducted a study to evaluate the nutritional composition of milk thistle obtained from three different locations. They found that the moisture content ranged from 5.01% to 6.27%, ash content ranged from 1.25% to 2.37%, fat content ranged from 19.74% to 23.19%, fiber content ranged from 4.39% to 7.4%, protein content ranged from 20.74% to 30.09%, and nitrogen-free extract ranged from 34.13% to 45.42%.

Apostol et al. (2017) explored the physicochemical properties and amino acid content of partially defatted milk thistle seeds. They reported that these seeds were a good source of protein (20.35%), lipids (11.69%), total carbohydrates (38.16%), and crude fiber (27.24%).

Bortlíková et al. (2019) investigated the application of milk thistle seed flour in functional biscuits. They found that milk thistle seed flour was rich in total dietary fiber (42.1%), proteins (20.1%), mineral compounds (5.5%), and fats (32.9%).

El-haak et al. (2015) studied the physical and chemical properties, as well as the nitrogenous compounds content, of milk thistle seeds grown in the north Delta of Egypt. They reported that milk thistle seeds were a good source of lipids (29.68%), true protein (25.25%), total carbohydrates (38.16%), crude fiber (29.95%), nitrogen-free extract (8.21%), and total nitrogen (4.41%). The seed proteins were found to contain high amounts of essential amino acids such as lysine, isoleucine, leucine, valine, and threonine.

Li et al. (2013) evaluated the basic physicochemical properties of protein fractions from *Silybum marianum* seeds. They found that the moisture content was 5.50%, ash content was 6.30%, fat content was 46.84%, fiber content was 6.46%, protein content was 19.85%, and nitrogen-free extract was 14.87% in S. marianum seed powders. Additionally, S. marianum seed defatted powders contained 37.52% protein.

Abd-El-hady (2019) studied the morphological, chemical characteristics, and antioxidant activity of wild milk thistle seeds grown in Egypt. The results showed that milk thistle seeds were mainly composed of protein (23.43%), moisture (4.36%), fat (26.14%), ash (4.92%), crude fiber (25.74%), and available carbohydrates (19.77%).

Khan et al. (2007) investigated the phytochemical properties of *Silybum marianum* seed oil. They reported that *Silybum marianum* seeds contained oil content of 26.05%, moisture content of 4.48%, ash content of 1.93%, crude fiber of 5.48%, carbohydrates content of 87.2%, and total proteins of 23%.

Zhu et al. (2013) analyzed the amino acid composition of *Silybum marianum* protein (SMP) and soy protein isolate (SPI). They found that SMP was mainly composed of protein (87.52%), moisture (4.2%), fat (0.68%), and ash (2.28%), while SPI was mainly composed of protein (93.18%), moisture (3.63%), fat (0.48%), and ash (2.80%).

Filho (2005) conducted a study on microbial spoilage of soy- and wheat-based canned meat analogues. The texturized soy protein was found to be primarily composed of protein (53.00%), fat (1.00%), ash (6%), carbohydrates (31.00%), fiber (3.00%), and moisture (6%).

Meng et al. (2023) investigated the relationship between raw material characteristics of soybean protein concentrate and the quality of textured vegetable protein. They found that soybean protein concentrate was mainly composed of protein (64.88-70.48%), fat (0.06-0.35%), ash (4.32-7.18%), and moisture (6.04-7.58%).

Hatamikia et al. (2019) studied the physicochemical, sensory, and microbial properties of plant-based protein products (meat-free burgers) formulated using various *Vicia ervilia* protein isolates. The texturized soy protein in their study was mainly composed of protein (91.52%), fat (0.25%), ash (2.67%), carbohydrates (1.85%), and moisture (3.75%). They also found that the product containing soy protein isolate was primarily composed of protein (14.18%), fat (12.48%), ash (2.23%), carbohydrates (12.69%), and moisture (58.23%). The product containing *Vicia ervilia* protein isolate was mainly composed of protein (15.34%), fat (12.69%), ash (2.91%), carbohydrates (11.82%), and moisture (56.45%).

Bakhsh et al. (2021) evaluated the rheological and sensory characteristics of a plant-based meat analog compared to beef and pork. The plant-based meat, which contained at least 74.90% textured vegetable protein (TVP), was mainly composed of protein (16.77%), fat (11.71%), moisture (51.53%), ash (3.23%), and fiber (6.87%).

Fresán et al. (2019) compared the sustainability and nutritional content of meat analogs from different protein sources. They found that soy-based products (containing at least 65% soy) were mainly composed of protein (24.96%), fat (6.63%), carbohydrates (20.31%), and fiber (6.35%). The wheat/soy-based products, in which both wheat and soy were below 65%, were mainly composed of protein (21.44%), fat (5.64%), carbohydrates (13.94%), and fiber (2.71%).

Curtain F. and Grafenauer S. 2019 in study to profile and compare plant-based meat substitutes (mimicking meat) with equivalent meat products. The result showed that the plant based burger mainly composed of protein (9.7%), fat (7.2%), carbohydrate (16.7%) and fiber (5.3%). Similar to Cole E. et al., 2021 in study to examination of the nutritional composition of alternative beef burgers available in the United States. The result showed that the plant based burger composed of protein (9%), fat (5%), carbohydrate (15%) and fiber (4%). Romão D. et al., 2022 in study of nutrients and main ingredients of vegan alternatives to meat products commercialized in Brazil. The result showed the plant based burger composed of protein (18.22%), fat (8.91%), carbohydrate (18.22%) and fiber (5.60%). Tonheim et al., 2022 in study comparing macronutrient content in substitutes with equivalent meat and dairy products. The result showed the plant based burger composed of protein (12%), fat (9.0%), carbohydrate (9.8%) and fiber (3.5%).

Curtain and Grafenauer (2019) conducted a study comparing plant-based meat substitutes with equivalent meat products. The meat burger in their study was mainly composed of protein (15.4%), fat (13.7%), carbohydrates (5.2%), and fiber (0.0%).

Romão et al. (2022) studied the nutrients and main ingredients of vegan alternatives to meat products commercialized in Brazil. They found that the animal burger was composed of protein (16.67%), fat (16.88%), carbohydrates (3.13%), and fiber (0.54%).

Contradicting these findings, Cole et al. (2021) examined the nutritional composition of alternative beef burgers available in the United States. They found that the meat burger was composed of protein (25%), fat (29%), carbohydrates (0-1%), and fiber (0.0%).

Yadav et al. (2015) conducted studies on the physicochemical properties and shelf life of developed chicken meat analogue rolls. The plant-based meat analogue in their study had protein, fat, ash, and moisture content of 15.79%, 8.20%, 3.75%, and 62.45%, respectively.

Mishal et al. (2022) developed a plant-based meat analogue and found that it had protein, fat, and moisture content of 22.32%, 2.46%, and 48.73%, respectively.

In a study by Domah et al. (2012), the effect of different types of soybean addition on the protein quality of processed kofta and burger was investigated. The results indicated that the protein content of meat and processed meat products decreased, ranging from 0.44 to 0.75g, during frozen storage for 6 months.

Similarly, Mohammed et al. (2021) studied the effect of frozen and refrozen storage of beef and chicken meats on inoculated microorganisms and meat quality. The moisture and protein content of both meat types decreased during nine months of frozen storage, while the fat and ash contents remained unchanged.

Hussein et al. (2020) conducted a study on the effect of freezing on the chemical composition and nutritional value of meat. The percentages of moisture, protein, and fat in each type of meat decreased during 4 months of storage, while the percentages of ash increased.

In another study by Mohammed et al. (2019), the effect of freeze and re-freeze on the chemical composition of beef and poultry meat during a storage period of 4.5 months was examined. The moisture content of both meat types increased during frozen storage, while the protein, fat, and ash contents decreased.

Overall, these studies highlight the changes in moisture, protein, fat, and ash contents of meat during frozen storage, with varying effects observed on different components depending on the specific study and storage duration.

In a study by Zhu et al. (2013), the digestibility of protein isolates from *Silybum marianum* (SMP and SPI) was investigated in vitro. The results showed that SMP was easily digested by pepsin plus trypsin, and it was much more easily digested by trypsin compared to SPI. SMP was found to be a good source of highly digestible protein, making it suitable for human consumption. It also had an excellent balance of essential amino acids, including relatively high levels of glutamic acid, arginine, leucine, and valine. The proportion of essential amino acids to the total amino acids (E/T ratio) was higher in SMP compared to SPI, indicating good nutritional quality based on the amino acid composition.

In a study by Mohammed et al. (2019), the antioxidant, antibacterial, and antifungal activities of different extracts of *Silybum marianum* were evaluated. The plant extracts showed greater effectiveness against gram-negative bacteria such as E. coli, P. aeruginosa, and A. baumannii. However, their antifungal activity was found to be low.

Abed et al. (2015) conducted a study on the antibacterial effect of flavonoids extracted from *Silybum marianum* seeds against common pathogenic bacteria. The chemical analysis revealed the presence of antimicrobial compounds such as terpenoids, flavonoids, and tannins in the seeds. The S. marianum seed extract demonstrated effectiveness against all bacterial species, with higher effectiveness against E. coli.

Yusuf et al. (2021) investigated the physicochemical properties, antioxidant, and antimicrobial activities of milk thistle *(Silybum marianum)* seed and leaf oil extracts. The seed oil extract exhibited stronger antifungal activity against A. versicolo, while the antibacterial activity against *E. coli* was relatively weaker. *S. aureus* was more susceptible to S. marianum seed oil extract than *E. coli.*

Oliveira et al. (2015) conducted an in vitro study on the antimicrobial and modulatory activity of silymarin and silibinin, natural products derived from *Silybum marianum*. Silymarin showed limited antimicrobial activity against the bacterial strains tested, while silibinin exhibited significant activity against *E.coli*. Both silymarin and silibinin showed similar antifungal activity against all strains.

In a study by Abdelazim (2017), the effect of silymarin as a natural antioxidant and its antimicrobial activity were evaluated. Different concentrations of silymarin exhibited antimicrobial activity against both gram-positive and gram-negative bacteria, as well as mold.

In a study by Hatamikia et al. (2019) investigating plant-based protein products formulated using *Vicia ervilia* protein isolates, the total plate count and yeast and mold count were found to be low in the products, with counts of 102 CFU/g and 10 CFU/g, respectively. This indicates good microbial quality and low levels of contamination in the plant-based protein products.

Contrasting with the above, Filho (2005) studied microbial spoilage of soy- and wheat-based canned meat analogues and observed an increase in total mesophilic aerobe counts and coliform counts during storage, with the highest counts observed in the sixth and seventh months, respectively. However, the yeast and mold counts decreased with increased storage time.

Tóth et al. (2021) studied microbial spoilage in pea protein-based products and found low aerobic colony counts in refrigerated pea-based products, ranging from log 10 CFU/g=1 to 2.3. Enterobacteriaceae were undetectable in all samples, and mold was observed in only one sample.

In a study by Baioumy and Abedelmaksoud (2021) examining the quality properties and storage stability of beef burgers with the addition of orange peels, the total plate count and yeast and mold count of the meat burgers increased with increased storage time, with the highest counts observed in the fourth month.

Regarding the peroxide value of *Silybum marianum* (milk thistle) seed oil, Yusuf et al. (2021) reported a low peroxide value of 1.10 meqKOH/g. Similarly, Dabbour et al. (2014) found a low peroxide value of 0.34 meqKOH/g for S. marianum seed oil. These values indicate low levels of oxidation in the seed oil.

In a study by Turk Baydir and Aşçıoğlu (2018) on the effects of antioxidant capacity and peroxide value on the oxidation stability of sunflower oil, it was reported that the peroxide value of sunflower oil ranged between 5.0 and 9.0 mEq/kg.

In a study by Elnour et al. (2022) on beef burgers enriched with olive oil, the PV of the burgers increased with storage time, ranging from 2.49 mEq/g initially to 4.63 mEq/g at the sixth month of storage. This indicates an increase in oxidative deterioration of the beef burgers during storage.

Contrasting with the above, Rahman et al. (2015) studied the physicochemical quality of beef hind limb during frozen storage and reported peroxide values ranging from

0.12 to 0.166 meq/kg. The initial thiobarbituric acid reactive substances (TBARS) value of fresh beef samples ranged from 0.272 to 0.542 mg MDA/kg beef.

Gheisari (2011) examined the correlation between acid, TBARS, peroxide, and iodine values, as well as catalase and glutathione peroxidase activities of chicken, cattle, and camel meat during refrigerated storage. The initial PV of cattle, camel, and chicken meat were reported as 0.12, 0.15, and 0.08 meq/kg, respectively. The initial TBARS values of cattle, camel, and chicken meat were 0.131, 0.204, and 0.066 µmol/kg, respectively.

In a study by Baioumy and Abedelmaksoud (2021) assessing lipid oxidation in a meat system with added phenolic-rich materials, the TBARS values of beef burgers increased from 0.23 to 0.68 mg MA/kg after 120 days of frozen storage. Similarly, Elama et al. (2017) investigated the use of oleuropein from olive leaf extract as a natural antioxidant in frozen hamburgers and observed an increase in TBARS values from 20.6 to 267.9 mg MA/kg sample after 6 months of frozen storage.

Regarding the use of milk thistle *(Silybum marianum)* in food products, Bortlíková et al. (2019) found that biscuits with 30% milk thistle flour were evaluated as having an undesirable taste and described as bitter with an unnatural taste. However, Krystyjan et al. (2022) reported that biscuits with a 5-15% addition of milk thistle were of good quality according to sensory analyses, while higher levels of milk thistle slightly deteriorated the quality parameters of the product.

In studies by Bakhsh et al. (2021) on plant-based meat analogs, sensory evaluations showed no significant differences in shape or color compared to beef and pork patties. The plant-based patties received a high score for color. However, they were found to have higher firmness compared to beef and pork. Another study by Bakhsh et al. (2021) explored the use of methylcellulose to modify the physicochemical, textural, and sensory characteristics of plant-based meat analogs, resulting in improved sensory scores without any beany essence.

In contrast, Marzena (2004) studied the effect of soy protein isolate on low-fat pork patties and found that it imparted a unique beany essence to the meat products, leading to a downgrade in sensory scores.

Chapter 3

Materials and methods:

3.1. Materials:

A. Material for prepared soy protein based meat analogues:

Milk thistle seeds were collected from Nablus Governorate, Palestine, soy protein flaks, wheat flour, water, methylcellulose, condiments, beet extract and salt.

B. Material for microbial examination:

Plate Count Agar (PCA), Violet Red Bile Glucose Agar (VRBGA), Sabouraud Dextrose Agar (SDA), Peptone water, Distilled water.

C. Material for chemical test:

Petroleum ether, Sulphuric acid (H_2SO_4), Potassium sulphate (K_2SO_4) , Copper sulphate ($CuSO_4$), Sodium hydroxide (NaOH), Boric acid (H_3BO_3), Ethanol (C_2H_5OH) , Methyl red , Bromocresol green dye, Hydrochloric acid (HCl), Alcohol, Phenol (C_6H_5OH), Acetic acid (CH3COOH), Chloroform ($CHCl_3$), Potassium iodide(KI), Sodium thiosulfate ($Na_2S_2O_3$), Thiobarbituric acid, Trichloroacetic acid, Starch , Distilled water. (All chemical martials purchased from Sigma Aldrich)

3.2 Methods:

3. 2.1. Preparation of plant burger (Meat free burger):

Milk thistle seed was grinded used an electric mill, then sieving milk thistle seed flour to obtain a uniform particle size then the flour was stored at 4 °C in dark until usage.

Textured soy protein was rehydrated with water (1:3 wt/wt) the mixture was heated at 70°C for 20 min using steam following the rehydration soy protein was washed ,dried and minced into particles using meat grinder. The other powder ingredients used in the formula were mixed (milk thistle seed flour, wheat flour, methylcellulose, condiments, salt and color agent (beet extract) and roasted for few minutes. Oil was added after which cold -4ºC water was mixed gradually to the mixture. The produced paste was molded subsequently in (circular) molds then the molded pastes placed in freezer in (-18ºC) for up to 6 months. Burger were formulated the in three formula.

Table 3.1 The % of ingredient of burger samples.

Formula	Common ingredient%	Different ingredient%
Sample1	28.2%water, 36% soy proteins flake 4% sunflowers oil, 0.5%salt, 0.5 condiments , 0.4% color , 2.4 methylcellulose	28% milk thistle seed flour
Sample2		14 % milk thistle seed flour+ 14% wheat flour
Sample3 (Control)		28% wheat flour

3.2.2. Microbial analysis:

3.2.2.1. Sample preparation:

25 grams of the food sample were mixed with 225 mL of peptone water and homogenized in a stomacher. A 4-fold serial dilution was carried out using 1 ml of the homogenized sample pipetted aseptically into 9 ml of peptone water contained in sterile, well-labeled test tubes arranged in a test tube rack. The diluted samples were pipetted onto Petri dishes in triplicate.

3.2.2.2. Total plate count (TPC):

The total plate count refers to the quantification of aerobic, mesophilic microorganisms that thrive under moderate temperatures and aerobic conditions, typically around 30 °C. This count encompasses both pathogenic and non-pathogenic organisms and serves as an indicator of the hygiene level in food production. Plate count agar is commonly employed to conduct total plate count analysis. It is important to note that plate count agar is a non-selective growth medium, meaning it supports the growth of various microorganisms without favoring specific types (Hanum G. et al., 2018).

To determine the microbial count in burger samples, 1 ml of each dilution was aseptically placed onto sterile Petri dishes. Subsequently, 15 ml of plate count agar that had been melted and cooled to 45 °C was poured onto the dishes. The agar was mixed thoroughly by rotating and tilting the dishes to ensure even distribution, and then the Petri dishes were incubated at 37 °C for a period of 24 to 48 hours. Following incubation, the colonies that developed on the agar surface were counted. The results were reported as logarithm base 10 of colony-forming units per gram (log10 CFU/g), representing the concentration of viable microorganisms present in the burger samples (Ismail K. & Belma D., 2002).

3.2.2.3.Yeast and mold:

Yeasts and molds play significant roles in various aspects of human life, including food production and industrial processes. They are utilized in the food industry for activities such as winemaking, single-cell protein production, brewing, baking, and vitamin synthesis. However, they can also cause spoilage in processed, preserved, and refrigerated foods when certain conditions are met. Therefore, it is important to enumerate and identify yeasts and molds in food to understand their contribution to different food systems and their potential for spoilage (Fung D., 2014)

To facilitate the enumeration and identification of yeasts and molds, Sabouraud Dextrose Agar is commonly used. This growth medium consists of enzymatic digestion of casein and animal tissues, which provide essential amino acids and nitrogenous compounds necessary for the growth of fungi and yeasts. Dextrose, a fermentable carbohydrate, is included in high concentrations to serve as a carbon and energy source for the microorganisms. Agar, a solidifying agent, is also added to the medium (Acharya T. & Hare J., 2022).

Burger samples (1 ml) of each dilution were transferred to sterile Petri dishes, and then 15 mL of SDA agar, which was cooled to 45 CO, was cast and mixed gently. The plates were incubated at room temperature for 5 days. After the incubation period, colonies were counted, and the results were expressed as log10 CFU/g..

3.2.2.4. Total coliform:

Coliform bacteria are defined as facultatively anaerobic, Gram-negative, non-spore-forming rods that ferment lactose vigorously to acid and gas at 35 ± 2 °C within 24 or

48 h (Halkman H.&Halkman A., 2014). Procedures to detect, enumerate, and presumptively identify coliforms are used in testing foods and dairy products. One method for performing the presumptive test for coliforms uses Violet Red Bile Agar (VRBA). Violet Red Bile Agar (VRBA) is a selective medium used to detect and enumerate lactose-fermenting coliform microorganisms.

Burger samples from various dilutions were aseptically transferred onto sterile Petri dishes, with each dish receiving 1 mL of the sample. Subsequently, 15 mL of violet-red bile glucose agar was poured into each Petri dish, ensuring thorough mixing, and left to solidify. The plates were then incubated at 37 °C for a period of 24 hours. Following the incubation period, the colonies present on the plates were counted, and the results were expressed as log10 CFU/g (Roth D. & Cambrel-Lenarz S., 2004; Kornacki J. & Gurtler J., 2001; AOAC, 2001).

3.2.3. Chemical analysis:

3.2.3.1. Determination of total fat content:

The total fat content was determined by FA-46 Fat Analyzer according to the method described by AOAC 945.38F (AOAC, 2006). FA-46 Fat Analyzer, designed in accordance with GB/T 14772-2008, can be used not only in measuring the fat content in food, diary product and feedstuff, but also in testing the soluble organic compound in detergent, medicine, petrochemicals, fiber products, soil and sewage etc.

The sample was ground, ensured that it was well homogenized and weighed 5 grams, and placed in a thimble filter. The thimble was put into the holder. Approximately 60 mL of petroleum ether was added to an extraction cup, dried well, weighed in the extraction cup (W1), and placed on a heating plate. After extraction was completed, the heating plate cooled, and the extraction cup was removed from the heating plate and put in the drying oven. After cooling it in the desiccator, the cup (W2) was weighted. Finally, the percentage of fat content was calculated.

Calculations:

%of the fat content = (Weight of cup with fat) -(Weight of empty cup)/ (sample weight) * 100 %.

3.2.3.2. Determination of protein content:

The protein total nitrogen content of the dried samples was analyzed using the micro-Kjeldahl technique. This method is based on the principles outlined in method 981.10 of the AOAC International (Latimer G., 2016; William H., 2000; Persson J., 2008; Puwastien P. et al., 2011).

In the digestion step, 1g of the dried samples was put into the Kjeldahl flask,and then 30 mL of concentrated sulfuric acid (95–97%), 0.4g of copper sulfate, and 3.5g of potassium sulfate were added. The mixture was heated slowly in a fume cupboard to prevent excessive frothing; then, the digestion was continued at 400°C for 3 hours until the color of the mixture changed to an iridescent blue color. The solution was left to cool down and diluted with distilled water to 100 mL. In the distillation step, 10 ml of the digested solution was added to 10 ml of NaOH (40%) and fixed to the distillation device. In the ammonia receiving flask, 10 mL of boric acid (4%) was added with three drops of a mixture of methyl red and Bromocresol Green dye. Collecting up to 30 mL in a receiving conical flask after the end of the distillation process.

In the titration step, the collected solution in the receiving conical flask was titrated with 0.1M HCL, and the titre was recorded, from which the amount of nitrogen content was measured according to this equation:

$$\%\text{Nitrogen} = [\{\text{mL (titre–B)} \times \text{M HCl} \times \text{dilution factor} \times 14.007\}/(\text{mg sample} \times 10)] \times 100 \quad \text{Eq.1}$$

Where: M HCL= Molarity of hydrochloric acid, B= Blank

$$\%P = \%\,N \times CF \quad \text{Eq.4}$$

Where: % P = Protein, % N = Nitrogen, CF = Conversion Factor

3.2.3.3. Determination of Ash content:

The ashing process involves the combustion of organic matter to determine the remaining inorganic matter in a sample. It is performed in two stages: firstly, to remove water and char the sample, and secondly, ashing at 550°C using a muffle furnace. This method is applicable to various food materials. The ash content was determined following the AOAC method 923.03, using a Box Muffle Furnace-SX2-4-10N (AOAC, 2006).

5 grams of sample was taken into the crucible of known weight, which was then dried and kept in a muffle furnace at 550°C for 3 h. The total ash content of the food sample was calculated:

Ash Content (%) = [Wt of ash / Wt of sample] * 100

3.2.3.4. Determination of fiber content:

The fiber content analysis was performed using the FIA-6-V2 Fiber Analyzer. This analyzer is suitable for determining the content of various fiber components, including Crude Fiber, acid/alkaline detergent fiber (ADF), neutral detergent fiber (NDF), acid detergent lignin (ADL), cellulose, and hemicellulose in raw materials and finished products of food, grain, and feed. The FIA-6-V2 Fiber Analyzer is designed specifically for fiber determination based on the method outlined by Soest.V. (1991).

The dry sample was ground and then sieved through a 0.45-mm mesh. Then a 3 g sample was put into a crucible of known weight. The instrument was preheated with water to almost 88 °C. Then a 150-ml H_2SO_4 solution was added to the crucible, and the solution was boiled within the set time. After the crucible was heated and filtrated, the crucible waste was discharged. A NaOH solution was added and filtrated. Then was added alcohol and filtrated (in cold digestion). Then it was put into the oven to dry, then weighed and calculated.

3.2.3.5. Determination of moisture content:

The moisture content was determined by Electronic moisture analyzer (MA 30-sartorius). The MA 30 moisture analyzer is a good instrument for detecting the moisture content of other agricultural and non-agricultural items due to its technique of analysis (drying of the sample and simultaneous detection of weight loss).

5 grams of each sample were weighed, and then the moisture analyzer was turned on with the temperature and time set at 105 °C for 15 minutes.

Calculations:

Moisture (%) = [Initial weight – Final weight/ Initial weight]*100

3.2.3.6 Determination of carbohydrate content:

The carbohydrate content was determined using the Phenol-Sulfuric Acid Assay, which was developed by Du bois M. et al. in 1956. This widely used method allows for the measurement of the total concentration of carbohydrates in a sample. It is suitable for lipid-free extracts obtained from cereals, seeds, and plants, as long as the sample is in solution. The Phenol-Sulfuric Acid Assay can be applied to both reducing and non-reducing sugars. In this method, the carbohydrates undergo a series of reactions in the presence of strong acids and heat, resulting in the formation of furan derivatives, such as furfuraldehyde and hydroxymethylfurfural. Previous studies conducted by BeMiller J. & Low N. (1998) and Pomeranz Y. & Meloan C. (1994) proved the reliability and accuracy of this assay.

Phenol, in an 80% solution, was added to a glass test tube containing a clear sample solution. Concentrated sulfuric acid was added in a rapid stream directly to the surface of the liquid in the test tube. The mixture was thoroughly combined using a vortex mixer and then permitted to stand for a sufficient time to allow for color development. Then the test tube was placed in a water bath at 25–30 °C for 20 minutes. The solution absorbance was read at 490 nm using a spectrophotometer. A calibration curve was constructed using the sugar. A stock 1 mg/ml aqueous sugar standard solution was used to prepare 5 or 6 standards ranging from 10 to 100 μg/ml. Each standard was subjected to the reaction procedure outlined above, and its absorbance was read at 490 nm.

3.2.3.7. Peroxide Value (PV):

The determination of peroxide value (PV) is a crucial test used to assess oxidation and rancidity in lipid-containing substances. It provides a measurement of peroxides and hydroperoxides that can form in unsaturated double bond lipids during the initial stages of oxidation. The peroxide value (PV) is expressed as the amount of reactive oxygen present in terms of milliequivalents (meq) of free iodine per kilogram of fat. The measurement is carried out by titrating the iodine released from potassium iodide with a solution of sodium thiosulfate. This method allows for the quantification of peroxide levels in the sample, providing valuable information about its oxidative stability. The importance of peroxide value determination in assessing lipid oxidation has been highlighted in studies such as Gheisari R. (2011), Dave D. & Ghaly E. (2011), and Pearson D. (1976).

The peroxide value was determined according to the method of sallam et al. (2004).The samples (3g) were weighted in a 250 ml glass stopper Erlenmeyer flask then it was heated for 3 min at 60°C in water bath to melt the fat. Aftar that the flask content added with for 3 min with 30 ml acetic acid –chloroform solution (CHCl3-CH3COOH) (3:2v/v) and thoroughly agitated to dissolve the fat. Whatman filter paper number 1 was used in filtration process to remove the particles. Saturated Kl solution (1 ml) was added to flask, then the flask was covered and put in the dark for 5 min. Distilled water (20ml) was added and the flask was shaken. Finally, the flask content was titrated with 0.01N $Na_2S_2O_3$ solution until light yellow color was appeared, after which 1.5% starch solution (1mL) was added as indicator and titration completed until colorless.

Calculation: $PV = (V_1\text{-}V_0) * N*1000/ W$

3.2.3.8. Thiobarbituric acid reactive substances (TBARS):

Thiobarbituric acid (TBA) reacts with malondialdehyde (MDA), forming a colored compound that can be detected through various methods such as spectrophotometry, chromatography, or image processing techniques. This reaction has been extensively studied in the literature, with references including Almroth B. et al. (2005), Seljeskog E. et al. (2006), and Xiong Z. et al. (2015). To account for the reactivity of TBA with other substances in biological samples, the term "Thiobarbituric acid reactive substances" (TBARS) has been widely adopted and is now commonly used (Sun Q. et al., 2001). TBARS is considered a standard marker for oxidative stress induced by lipid peroxidation (Tsai M. & Huang T., 2015). In the context of meat samples, the measurement of thiobarbituric acid reactive substances (TBARS) has been employed to monitor lipid oxidation. The TBARS values were determined on a fat basis using a slightly modified method based on the procedure outlined by Aytul (2010).

Meat sample (5 g) was homogenized with 20 mL tri-chloroacetic acid solution (15% w/v) and then centrifuged at $3000gg$ for 10min.The supernatant (2 mL) was mixed with 2 mL thiobarbituric acid solution (0.1% w/v in double distillated water) followed by heating in a water bath at 100°C for 30 min and then cooling to room temperature. Therefore, TBARS were extracted in chilled atmosphere. The absorbance of each extract was measured at 520 nm in a spectrophotometer (spec 1650PC, Shimadzu, Japan). Malondialdehyde (1,1,3,3- tetraethoxypropane) was used to develop the standard curve for TBARS assay. TBARS values were reported as mg of malonaldehyde per kg of burger.

3.2.4. Sensory evaluation:

Eight panelists with some experience, 6 man and 2 women, between 22 and 45 years ago, belonged to the Department of Food Processing at Al-Quds University, were trained in sensory analysis for at least 3 hours to identify the descriptors that best fitted the product in terms of appearance ,odor ,taste and texture. The list of the sensory attributes, definitions, and scale is reported in Table 3.2. Panelists were evaluated the samples on structured scale ranging from 0 to 10 points.

5 g of each sample were grilled, then placed in a glass container, codified with a three-digit alphanumeric code, and then presented to each panelist for the descriptive sensory analysis. Each panelist carried out the sensory evaluation in a sensory room according to ISO 8589:2007.

Table 3.2. Sensory attributes, definition, and scale anchors for meat analogues (scale 0–10).

	Attribute	Definition	Scale Anchors
Appearance	**Color**	Perceived color tone	0-brown , 3- pink , 10-bright red
Odor	**Overall intensity**	Overall odor intensity of the sample	0 –not perceived odor,10-very intense odor
	Meat-like	Perceived similarities with meat	0-not resembling to meat10-very similar to meat
Taste	**Overall intensity**	Overall taste intensity of the sample	0 –not perceived taste;10-very intense taste
	Cereals	Association with cereal	0-none;2-very mild;4-mild;6moderate;8-intense;10-very intense
	Legumes	Association with legumes	
	Off-taste intensity	Non-characteristic tastes (chemical, rancid, metallic, etc.)	

Texture	**Springiness**	Rate to which the sample recovers to its initial condition after pressing it with fingers	0-not recovering; 3 recovers slightly ; 10-recovers completely
	Hardness	The force required to compress the sample using teeth	0-easily compressible; 8 hardly compressible; 10-not compressible
	Chewiness	Effort required to chew the sample until it can be swallowed	0-no chews needed ; 8-moderately hard to chew;10-requires lot of chews to masticate food

3.2.5. Statistical analysis:

All microbiological counts and chemical tests were replicated three times, and the data are presented as mean ± standard deviation; sensory evaluations are presented as median ± standard deviation by Microsoft excel. The effects of addition milk thistle seed flour were analyzed and the obtained data were subjected to analysis of variance (ANOVA) accompanied with Duncan test using SPSS software (SPSS Inc., Chicago) to identify the significance ($p < 0.05$) between means of treatments.

Chapter 4.

Results

4.1. Microbial test:

4.1.1. Total plate count (TPC):

Figure 4.1 shows the mean microbial load values for TPC from the three plant burger samples before and during 6 months of cold storage. Generally, the result showed that all the samples, in terms of TPC values, decreased with increasing time of freezing, especially after four and six months. At zero time and the second month of storage, all samples exhibit a high value; also, sample 1 has a higher value than samples 2 and 3. However, after six months of storage, the TPC value of sample 1 (1±1 CFU/g) became less than that of sample 2 (1.67±0.57 CFU/g) and sample 3 (2.3±0.57 CFU/g).

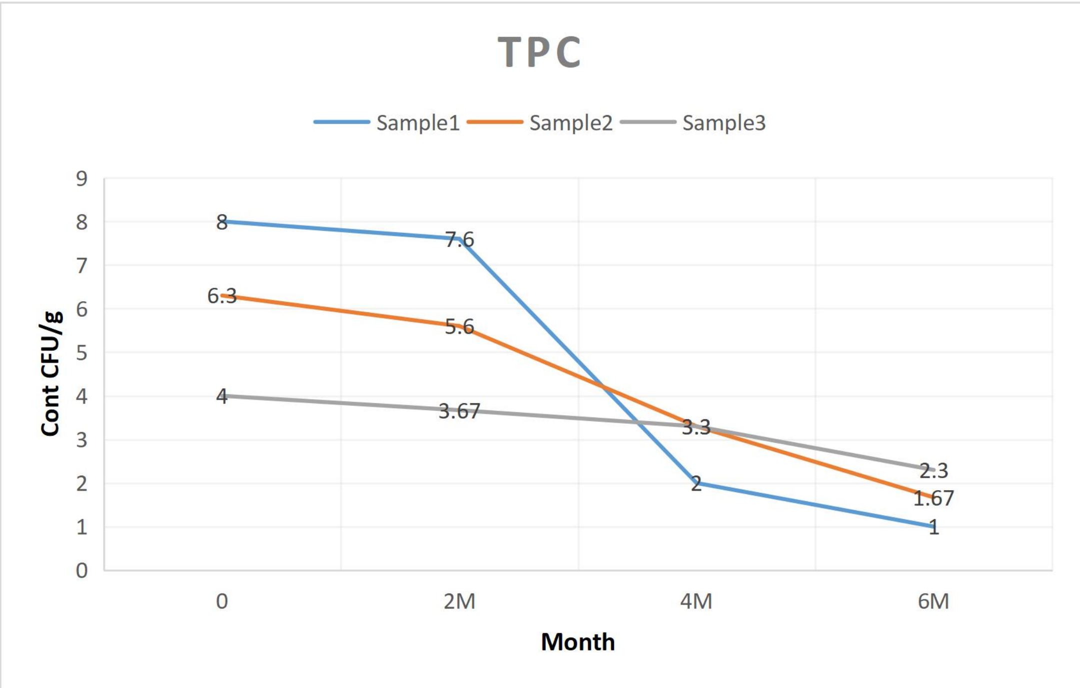


Figure 4.1: Result of TPC (CFU/g) of samples during 6 months of storage. Sample 1: contains 28% MTSF; Sample 2: contains 14% MTSF and 14% WF; Sample 3: contains 28% WF.

4.1.2. Yeast and mold:

Figure 4.2 shows the microbial load values of yeast and mold count from the three plant burger samples before and during 6 months of cold storage. The yeast and mold count decreased obviously in all samples as the time of freezing increased, apart from this, the highest count was observed in sample 2 in the first count (5.3±1.52 CFU/g). However in the sixth month, the highest count was observed in sample 1 (3±1.4 CFU/g), whereas sample 3 (2±1 CFU/g) has the lowest count in the sixth month.

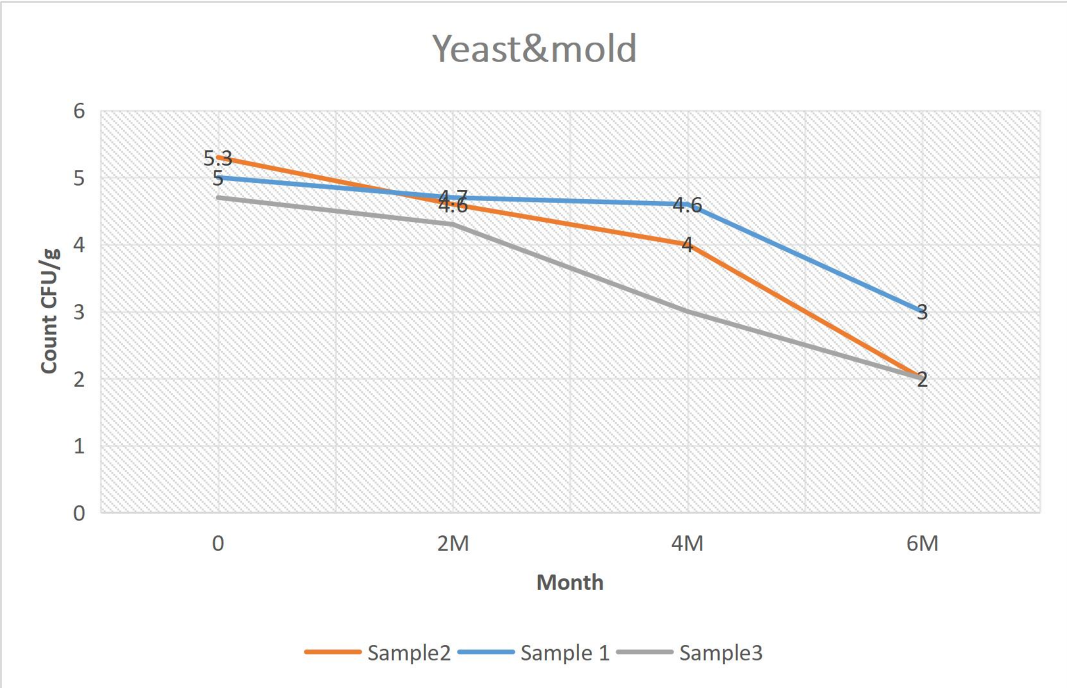


Figure 4.2: Result of yeast & mold count (CFU/g) of samples during 6 months of storage. Sample 1: contains 28% MTSF; Sample 2: contains 14% MTSF and 14% WF; Sample 3: contains 28% WF

4.1.3 Total coliform:

Figure 4.3 shows the microbial load values of total coliform count from the three plant burger samples before and during 6 months of cold storage.

The result showed that the total coliform count decreased obviously in all samples as the time of freezing storage increased. The highest coliform count was observed in sample 2 (7.67±1.52 CFU/g) in the all test. The lowest coliform count observed in Sample 1 (2±0 CFU/g) in the sixth month**.**

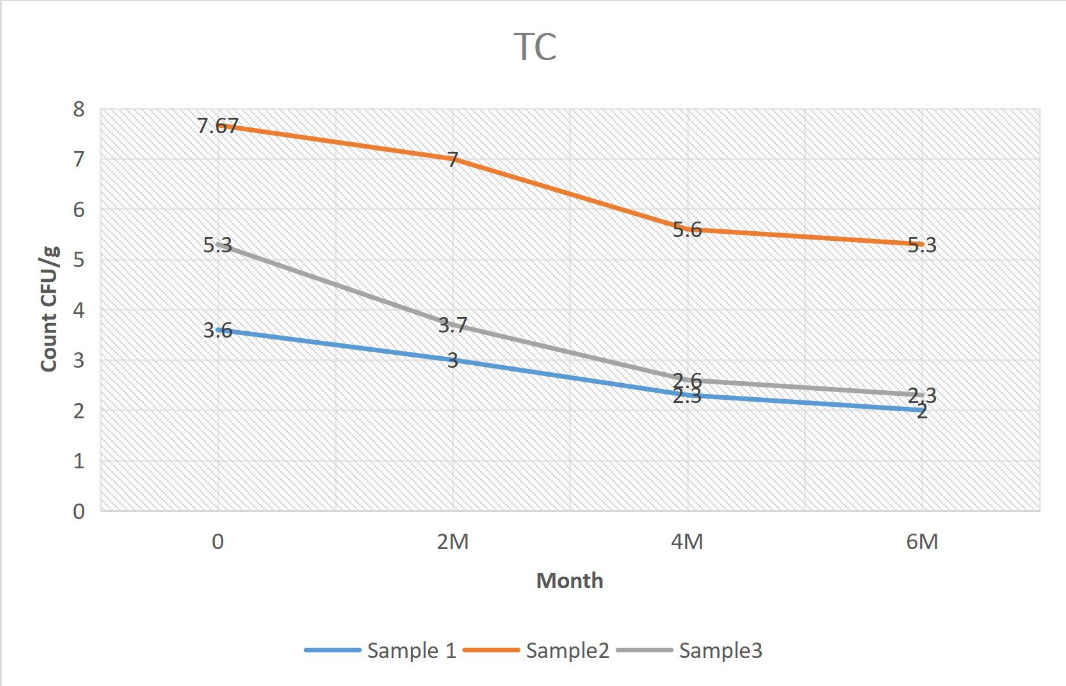


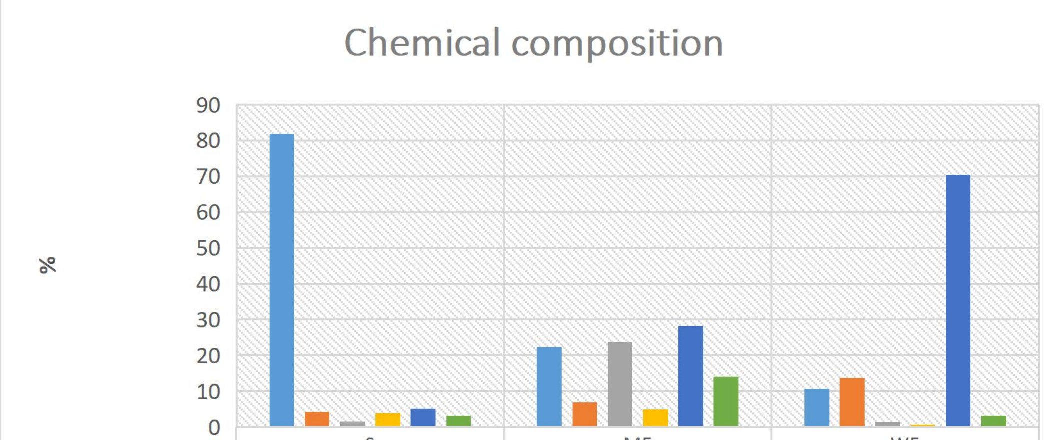
Figure 4.3: Result of Total coliform count (CFU/g) of samples during 6 months of storage. Sample 1: contains 28% MTSF, Sample 2: contains 14% MTSF and 14% WF, Sample 3: contains 28% WF

4.2. Chemical test:

4.2.1. Chemical composition:

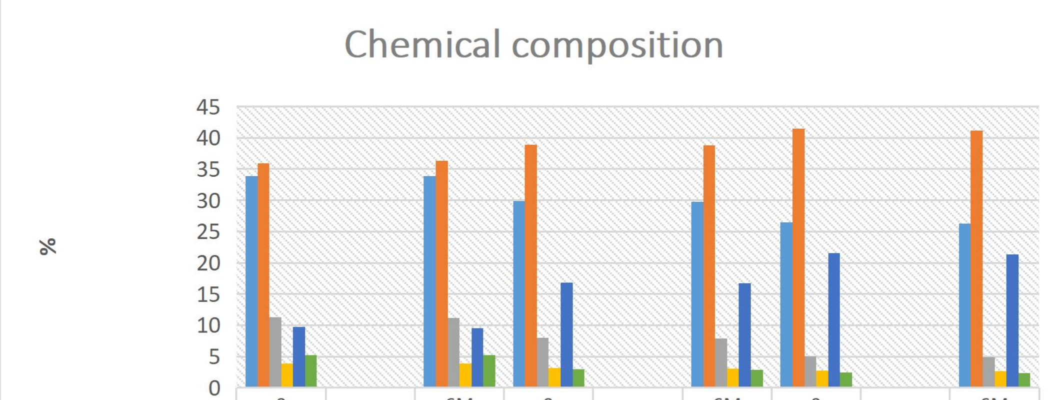
Without a doubt, the protein content in soy protein flakes (81.8±0.43) is the highest. Importantly, milk thistle flour contains more protein (22.32±0.17) than wheat flour (10.7±0.25). On the other hand, the highest content of fat (23.65±0.13), ash (4.92±0.21) and fiber (14.07±0.16) were observed in milk thistle flour, while the highest content of carbohydrates (70.35±0.61) and moisture (13.7±0.09) were found in wheat flour. (Figure 4.4).

Before and after freezing, soy plant-based meat burgers produced from milk thistle seed flour (sample 1) showed higher protein, fat, ash, and fiber content than those produced from wheat flour (sample 3) and a mixture of two flours (sample 3). While sample 3 was characterized by the highest carbohydrate content, followed by sample 2. Additionally, the results demonstrated that all samples' protein, fat, ash, carbohydrates, and fiber contents significantly ($p > 0.05$) reduced with increasing storage duration. Before the samples were frozen, sample 3 (41.45± 0.2) showed a higher moisture content than samples 1 (35.92 ± 0.12) and 2 (38.89± 0.01). However, after six months of freezing, samples 2 and 3 showed decreased moisture contents, while sample 1 (36.35± 0.09) showed increased moisture contents.



	S	MF	WF
Protein	81.8	22.32	10.7
Moisture	4.3	6.9	13.7
Fat	1.6	23.65	1.3
Ash	3.9	4.92	0.7
Carbohydrates	5.2	28.23	70.35
Fiber	3.2	14.07	3.07

Figure4.4: Chemical composition of main raw materials. SP: Soy proteins flakes, MTSF: Milk thistle seed flour, WF: Wheat flour**.**



	Sample 1			Sample 2			Sample 3		
	0		6M	0		6M	0		6M
Protein	33.9		33.83	29.88		29.79	26.49		26.31
Moisture	35.92		36.35	38.89		38.75	41.45		41.2
Fat	11.3		11.22	7.99		7.92	5.01		4.89
Ash	3.87		3.83	3.15		3.1	2.76		2.63
Carbohydrates	9.72		9.53	16.82		16.73	21.54		21.36
Fiber	5.19		5.18	2.94		2.81	2.41		2.36

Figure 4.5: Chemical composition of plant-based burger samples before and to the end of experiment (6M). Sample 1: contains 28% MTSF, Sample 2: contains 14% MTSF and 14% WF, Sample 3: contains 28% WF

4.2.2. Peroxide value:

The peroxide values of soy plant-based meat during freezing storage are presented in Figure 4.6. The results showed that peroxide values increased significantly as the time of freezing increased, and significant differences (p> 0.05) between samples were shown in all months of storage. Obviously, the highest values were observed in sample 1 (which contains 28% milk thistle flour) during the four months of storage. In the sixth month of storage, sample 1 showed the lowest peroxide value.

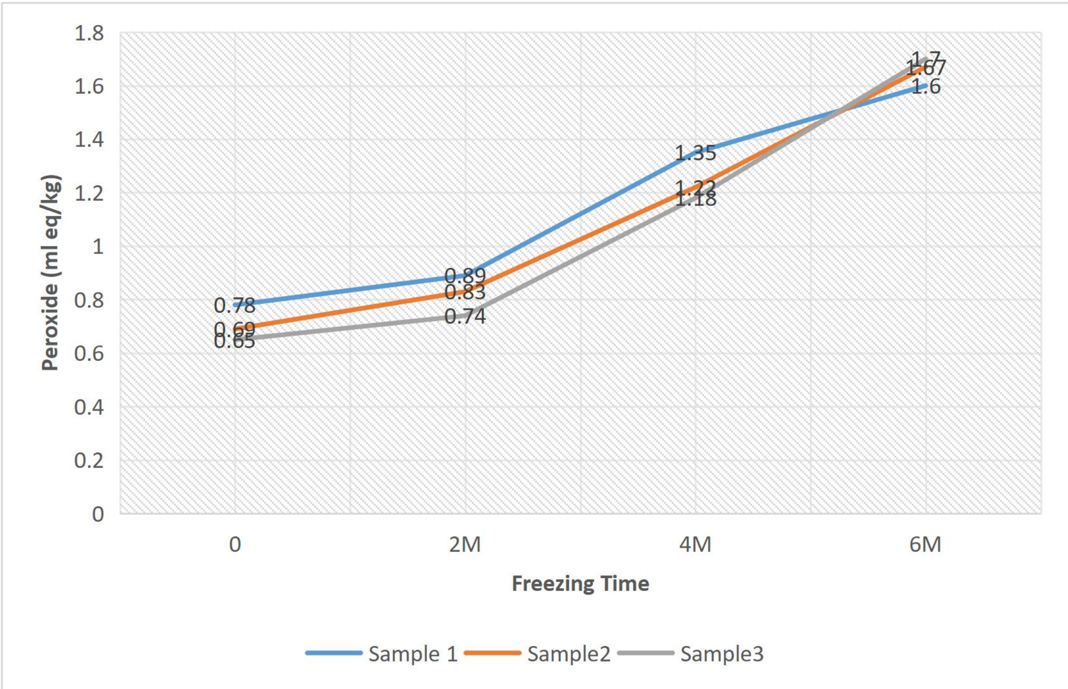


Figure 4.6: Peroxide value (mEq/kg) of plant-based burger samples during 6 months of frozen storage. Sample 1: contains 28% MTSF, Sample 2: contains 14% MTSF and 14% WF, Sample 3: contains 28% WF

4.2.3- TBARs value:

The TBARs values of soy protein meat during freezing storage are presented in Figure 4.7. The results showed that TBARs values increased significantly (p> 0.05) as the time of freezing increased; an observed difference between samples 3 was shown during the storage period. Obviously, the lowest values were observed in sample 1 during the six months of storage.

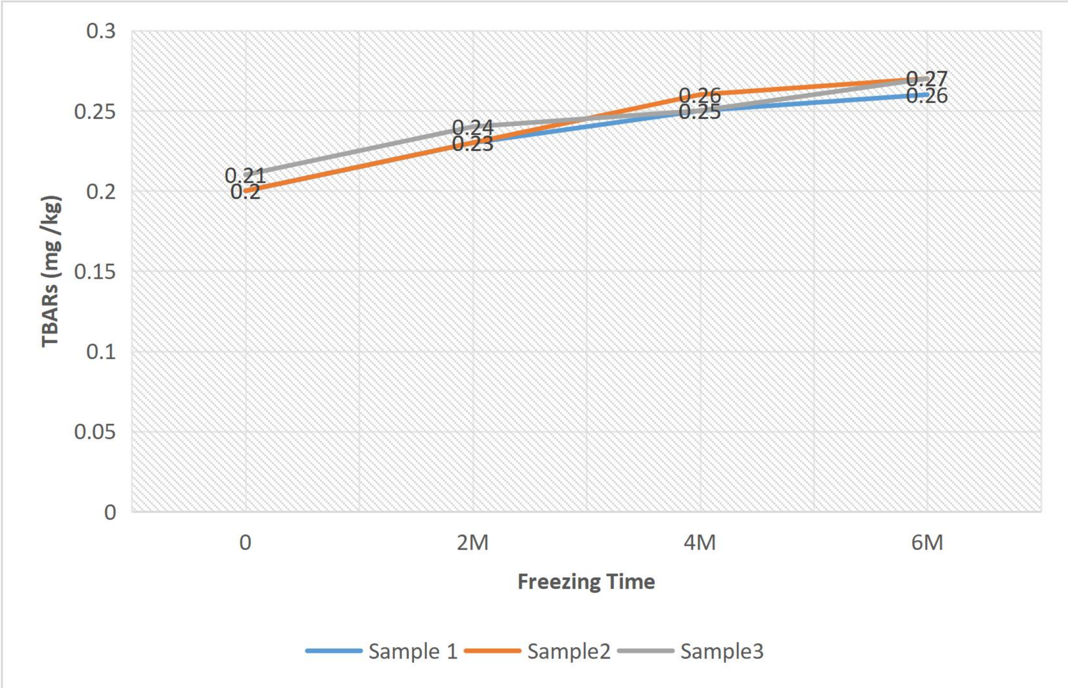


Figure 4.7: TBARs value (mg MAD/ kg meat) of plant-based burger samples during 6 months of frozen storage. Sample 1: contains 28% MTSF, Sample 2: contains 14% MTSF and 14% WF, Sample 3: contains 28% WF

4.3. Sensory evaluation:

The attributes of all soy protein meat samples were clearly distinguished by panelists according to the anchors reported in Table 3.2.

As can be observed in Table 4.8, the use of milk thistle seed in soy plant-based meat led to products with a brown color, whereas the use of milk thistle flour and wheat flour in soy protein meat led to products with a brown-pink color. But when wheat flour was used, this led to the presence of a pink-red color. Sample 1 gave the highest overall odor intensity (5.8±0.42) than samples 2 and 3. whereas a meat-like odor was not observed in all samples. The best overall taste intensity was detected in sample 3 more than in sample 2, while the cereal taste was highly observed in sample 1, but the legume taste was highly scored in sample 3. The panelists did not sense saltiness or an off-taste in any samples. Additionally, the panelists was evaluated springiness and hardness attributes in sample 3 more than in samples 1 and 2. Chewiness was observed in sample 1 more than in samples 2 and 3.

Table 4.8 Sensory analysis of soy plant-based burger samples.

	Attribute	Sample 1	Sample 2	Sample3
Appearance	**Color**	1.89±0.37	3.12±0.34	6.35±0.42
Odor	**Overall intensity**	5.8±0.42	4.35±1.47	5.2±0.95
	Meat-like	0±0	0±0	0.00± 0.00
Taste	**Overall intensity**	5.75±0.83	3.86±0.95	2.43±0.94
	Cereals	2±0.57	1.90±0.55	1.75±0.54
	Legumes	1.3±63	1.0±0.34	1.9±0.49
	Saltiness	0±0	0±0	0±0
	Off-taste intensity	0±0	0±0	0±0
Texture	**Springiness**	3.43±0.94	4.25±0.95	4.75±0.83
	Hardness	3.20±0.97	3.12±0.80	3.1±0.62
	Chewiness	7.5±0.75	4.1±0.67	3±0.72

Values are indicated as median ± standard deviation. Sample 1: contains 28% MTSF, Sample 2: contains 14% MTSF and 14% WF, Sample 3: contains 28% WF.

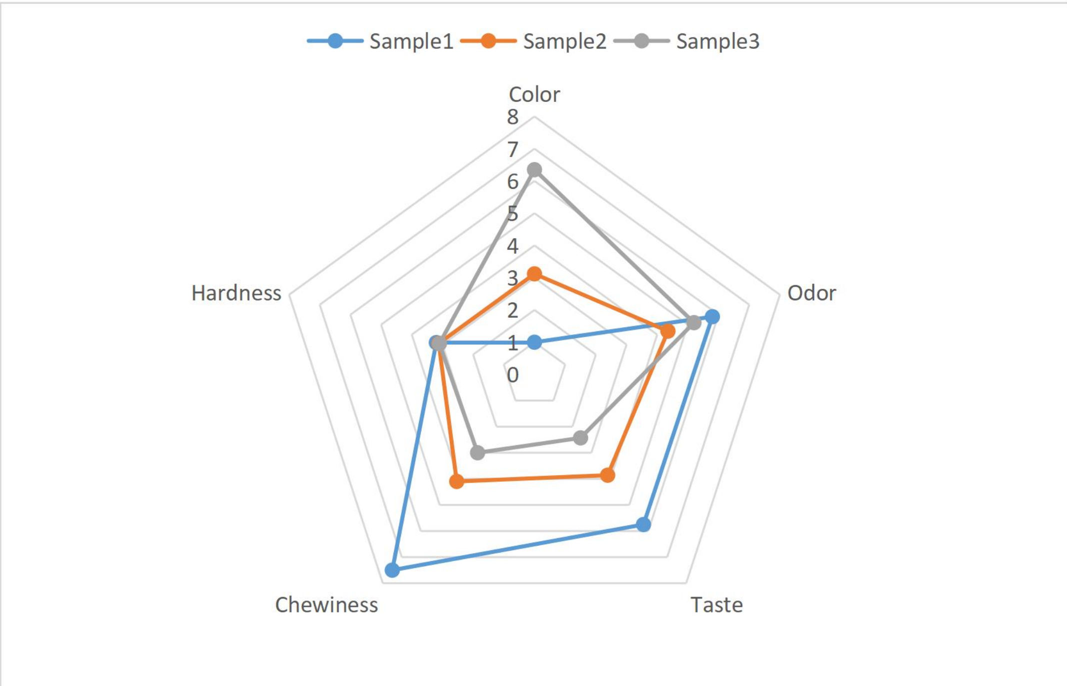


Figure 4.8: Sensory evaluation soy plant-based burger samples. Sample 1: contains 28% MTSF; Sample 2: contains 14% MTSF and 14% WF; Sample 3: contains 28% WF.

Chapter 5

Discussion

5.1. Microbial analysis:

5.1.1. Total plate count:

All of the samples have significant moisture contents, as seen in Figure 4.5, which could raise the microbial counts. This observation is consistent with a study by Andrew et al. (2021), which examined microbial spoilage of plant-based meat analogues and found that high water content and water activity in protein-based raw materials can promote bacterial proliferation. Similarly, in studies focusing on animal meat, such as those conducted by Berger et al. (2018) and Hamad et al. (2019), it has been noted that high moisture content is one of several factors favorable for microbial growth.

The total plate count of all burger samples decreased as the duration of frozen storage increased. Freezing is known to inhibit the growth of microorganisms to some extent, but complete elimination of microorganisms, especially spore-forming bacteria, is not always achieved (Gouvêa, Mendonça, Lopez, & Batalha, 2016).

The inclusion of milk thistle seed flour, which has antibacterial activity, may have contributed to the reduce in the total plate counts of sample 1 (which contains 28% MTSF), which reduced from 8-1 CFU/g to 1-1 CFU/g. This antimicrobial activity can be attributed to the high content of flavonoids in milk thistle seeds (Abe et al., 2015). The antibacterial activity of flavonoids can be explained by the harmful effect of this compound towards microorganism, such effect , could be the establishment by hydrogen bonds with the cell walls proteins or enzymes, the chelation of metal ions, inhibition of bacterial metabolism, sequestration of substances necessary for the growth of bacteria. In addition, the β ring of flavonoids is important in the intercalation with nucleic acids, thus inhibits DNA and RNA synthesis.

5.1.2. Yeast and mold:

The presence of molds and yeasts in food was usually related to raw materials or post process contamination, therefore mold and yeast counts can be used as a hygiene indicator primarily in the case of plant-based products.

The yeast and mold count of all burger samples decreased during the duration of frozen storage, which is consistent with a study conducted by Filho (2005) on canned plant-based burgers over a period of 12 months.

It is important to note that the inclusion of milk thistle flour did not have a significant effect on the yeast and mold count. This finding aligns with the observations made by Mohammed et al. (2019) and Oliveira et al. (2015), who reported that the antifungal activity of milk thistle extract was low against yeast and mold growth. However, it should be mentioned that Yusuf et al. (2021) observed a stronger antifungal activity of milk thistle seeds.

5.1.3 Total coliform:

In general, data in Figure 4.3, indicated that all burger samples were contaminated with coliform in initial counts.

This increase might be result from the favorable temperature and time conditions present during the preparation stages prior to retorting and a cross contamination might occurred from equipment's used for preparation. However, at the end of hydration step (70°C for 20 min), the temperature of the soy protein was approximately 45°C, which could be an ideal temperature for coliform development. Hydration of the soy protein is required for product quality and can promote microbial growth. The ground soy protein was stored for approximately 2 h prior to mixing the ingredients.

Although post-disinfection surface specifications for coliforms are not commonly available (because these values are likely to depend upon the environment, product and process) target values of <2.5 CFU/cm^2 have been suggested and are generally attainable for a range of surfaces (Moore et al., 2002). It should be noted that the presence of very low concentrations (<2.0 log10 CFU/cm^2) of bacteria on surfaces may still be a significant risk factor for contamination thus emphasizing the importance of effective cleaning and disinfection procedures.

Maintaining sanitary conditions during the processing stages is crucial for minimizing the microbial contamination of the final product, as highlighted by Filho et al. (2005).

The decline that occurred in total coliform counts of sample 1 (which contains 28% MTSF) from 3.6±0.57 CFU/g to 2±0 CFU/g may be related to the high content of milk thistle seed flour in this sample, which possesses antimicrobial activity against coliform bacteria. This finding aligns with several previous studies that have demonstrated the effectiveness of milk thistle against coliform bacteria, particularly *E. coli*. These studies include Mohammed et al. (2019), Abed et al. (2015), and Oliveira et al. (2015).

The antimicrobial properties of milk thistle, especially its activity against coliform bacteria, have been well-documented. These findings further support the potential use of milk thistle as a natural antimicrobial agent in food products.

5.2 Chemical analysis:

5.2.1. Chemical composition:

A. Chemical composition of raw materials:

As seen in Figure 4.4, the chemical composition of milk thistle seed flour used in soy protein burgers shows that it is a rich source of protein, fat, fiber, and ash since its contents are 22.32%, 23.65%, 14.07%, and 4.92%, respectively. Moisture is 6.90%, and carbohydrate is 28.23%. Whereas wheat flour used in soy protein burgers is a rich source of carbohydrate (70.35%) and moisture (13.7%). The results are consistent with the previous work of Bortlíková et al., 2019, who investigated milk thistle seed flour used in biscuit formulation as a rich source of protein, fat, fiber, and ash, since content was 20.1%, 32.9%, 42.1%, and 5.5%, respectively, whereas wheat flour used in biscuit formulation was a rich source of carbohydrate (67.7%) and moisture (8.9%).

The protein content of milk thistle seed flour in the present study is consistent with the findings of Aziz et al. (2020), Apostol et al. (2017), Khan et al. (2007), and Abd-El-hady (2019), indicating a high convergence of results.

However, there are some contrasting findings in the literature. El-haak et al. (2015) reported a protein content of 25.25% in milk thistle seeds, which is higher than the values observed in the present study. On the other hand, Li et al. (2013) found a lower protein content of 19.85% in milk thistle seeds.

In comparison to previous studies, the cured fiber content of milk thistle seeds in the present research differs from the findings of El-haak et al. (2015), Apostol et al. (2017), and Bortlková et al. (2019), who reported higher cured fiber content. Conversely, Aziz et al. (2020) and Li et al. (2013) reported lower cured fiber content in milk thistle seeds.

Regarding the ash content, the findings of the present study align with Abd-El-hady (2019), but differ from the results reported by Li et al. (2013) and Aziz et al. (2020), who reported the highest and lowest ash content, respectively.

The fat content in the current study is similar to the oil content reported by Aziz et al. (2020), whereas Apostol et al. (2017) reported a lower fat content and Li et al. (2013) reported a significantly higher fat content compared to the present results.

The moisture content findings are consistent with Aziz et al. (2020), but differ from Khan et al. (2007) and Abd-El-hady (2019), who reported lower moisture content compared to the present study.

The carbohydrate content of milk thistle seed flour in the present study contradicts the findings of Apostol et al. (2017), Abd-El-hady (2019), and El-haak et al. (2015).

These variations in composition can be attributed to various factors such as plant variety, climate, ripening stage, seed harvesting period, and location. It is important to consider these factors when comparing the results of different studies on milk thistle seeds.

B. Chemical composition of soy plant-based burger samples:

As seen in Figure 4.5, all samples contain a high level of protein. The final burger's protein rate was directly associated with the raw materials constituting the burger.

The burger sample with 28% milk thistle seed flour exhibited the highest protein content (33.90%). This finding contradicts studies on plant-based meat, such as Hatamikia et al. (2011) and Bakhsh et al. (2021), which reported lower protein content in soy-based products (14.18% and 16.77% respectively). Similarly, the protein content of all burger samples in the current study was higher than that of animal meat burgers, as reported by Curtain and Grafenauer (2019) and Romão et al. (2022) (15.4% and 16.67% respectively).

The high protein level in sample 1 can be attributed to the large percentage (28%) of milk thistle seed flour used, which has a high protein content (22.32%). Additionally, the thermal stability of *Silybum marianum* protein, as stated by Zhu et al. (2013), contributes to its resistance to denaturation.

Sample 1 also contained the lowest amount of carbohydrates (9.72%). This finding is consistent with Tonheim et al. (2022), who reported low carbohydrate content in plant-based burgers (9.8%). However, Fresán et al. (2019) found that soy-based products are rich sources of carbohydrates (20.31%).

In comparison to their animal counterparts, plant-based meat substitutes generally have higher concentrations of carbohydrates, as reported by Cole et al. (2021), Romão et al. (2022), Curtain and Grafenauer (2019), and Tonheim et al. (2022). These studies indicated carbohydrate values ranging from 7-15 g/100 g in plant-based meat substitutes, compared to 0-3 g/100 g in meat products.

The fiber content of sample 1 in the present study aligns with the findings of Curtain F. and Grafenauer (2019), indicating that meat substitutes with milk thistle seed flour can be a good source of dietary fiber. This is in contrast to the low fiber content (2.71%) reported by Fresán et al. (2019) for wheat-soy-based meat. The inclusion of milk thistle seed flour in meat substitutes provides an opportunity to increase fiber consumption, particularly in Western diets where fiber intake is typically low. Fiber also contributes to the technological and sensory characteristics of food products by retaining water and enhancing texture and resistance to breakage, similar to meat products.

The fat content of sample 1 (11.30%) in the current study aligns with the findings of Bakhsh et al. (2021). Fat plays a crucial role in sensory characteristics such as lubricity, palatability, aftertaste, and shelf life. These characteristics are important for the market acceptance and overall desirability of food products. Although plant-based products generally have lower amounts of natural fat compared to animal-based products, manufacturing fat-free meat substitutes is impractical as it would negatively impact their sensory characteristics, making them less appealing to consumers.

The moisture content was observed to be higher in sample 3 (41.39%) compared with other samples. Moisture level is an important consideration since it impacts the physical properties, nutritional composition, and shelf life of the produced product, all of which are quality attributes.

All meat analogue samples' protein, fat, carbohydrate, fiber, and ash contents don't change noticeably over the duration of a frozen storage period.

As the result showed, the moisture contents in samples 2 and 3 dropped after six months of frozen storage. Whereas samples that contain 28% MTSF exhibit a noticeable rise in moisture content after six months of storage of 0.43%. The study by Mohammed H. et al. (2019), which noted that the moisture content in frozen meat samples increased with storage time, However, there is a discrepancy with the findings of Mohammed H. et al. (2021) and Hussein H. et al. (2020), who reported that the moisture content of frozen meat samples decreased during storage.

5.2.2. Peroxide values:

In the present study, the PV was utilized to assess the antioxidant activity of milk thistle in plant burgers. The initial PV values of all burger samples ranged from 0.65 to 0.78 mEq/kg, which were higher than those reported by Rahman et al. (2015) for their sample. In comparison, Elnour et al. (2022) reported a PV of 2.49 mEq/g for a beef burger enriched with olive, indicating some degree of primary oxidation had occurred before storage, potentially due to the heat processing of soy protein flakes.

As shown in Figure 4.6, the peroxide values of all burger samples increased with the duration of frozen storage. While frozen storage can slow down the oxidation process, it cannot completely halt oxidation. In the sixth month of storage, sample 1 (containing 28% milk thistle seed flour) exhibited the lowest PV of 1.60 mEq/kg among the three samples, whereas sample 3 (the control sample) had the highest PV of 1.70 mEq/kg. This observation may be attributed to the antioxidant activity of milk thistle, as noted by Yusuf et al. (2021), who reported low peroxide and acid values in milk thistle, indicating low oxidation rancidity and high biological activities.

Milk thistle seed is known to be a rich source of vitamin E and polyunsaturated fatty acids, particularly linoleic and oleic acids. The presence of these highly unsaturated fats contributes to the low peroxide levels, even after extensive oxidation, as peroxides formed from unsaturated fats are initially highly unstable and rapidly react to form secondary oxidation products (Gotoh and Wada , 2006; Levermore, 2004).

5.2.3. TBARs:

In this experiment, TBARS values ranged from 0.20 to 0.27 mg/kg, which are lower than the acceptance limit of TBARS for rancidity (1.0 mg/kg). According to Rahman et al. (2015), the TBARS value of a fresh beef sample was 0.272 mg MDA/kg.

In the current study, it was observed that TBARs (thiobarbituric acid reactive substances) values increased with the duration of storage. This finding aligns with previous research conducted by Devatkal et al. (2004) and Rajkumar et al. (2004), who reported a natural tendency of TBARs values to increase during refrigerated and frozen states of meat and meat products.

Sample 1 (with 28% MTSF) in the sixth month had the lowest TBARs values of 0.26 mEq/kg compared with the other two samples.

5.3. Sensory evaluation:

As it can be observed in Table 4.8, the analysis of the obtained result indicated regard of color and texture that there was significant difference between samples .

The findings from the present study indicate that the sample containing 28% milk thistle seed flour showed poor color evolution, which is consistent with the observations made by Veronika et al. (2019) in their evaluation of biscuits with milk thistle seed flour. The addition of milk thistle seed flour may have contributed to the undesirable color changes in the product.

The overall taste intensity of sample 1 was higher than that of the other samples. This could be attributed to the presence of milk thistle seed coats, which are high in fiber content, including lignin (Mohamed et al., 2016). Changes in taste perception can occur due to the presence of specific components in the ingredients used.

The use of plant proteins in meat analogues can lead to the generation of volatile compounds from lipid oxidation, resulting in unappealing odors and flavors. This is a common disadvantage associated with plant-based proteins in meat substitutes, as mentioned by Asgar et al. (2010).

Sample 1, which has the highest fiber content, also exhibited higher scores for hardness and chewiness. Excessive use of dietary fiber in these products can contribute to negative textural characteristics, such as increased rigidity and the need for more chewing.

The high protein content in meat analogues produced from protein isolates can lead to the formation of a dense fibrous network due to covalent isopeptide cross-linking. This can result in a harder texture. Additionally, plant-derived proteins, such as soy protein, have aggregation abilities that can contribute to stretchy, rubbery, and chewy sensations in meat substitutes, as noted by Bakhsh et al. (2021) and Wi et al. (2020).

The stable structure observed in sample 3 may be attributed to its high carbohydrate content. Carbohydrates play a role in improving the interaction between protein, lipid, and water components in processed food systems, thereby influencing the overall texture and structure of the product.

These findings highlight the importance of ingredient composition and formulation in achieving desirable sensory attributes in meat analogues and the potential challenges associated with the use of certain ingredients.

Chapter 6

Conclusion:

6.1Microbial analysis:

The results of this part show a higher initial microbial load for all soy plant based meat analogues. This indicated that the raw materials for plant-based meat analogues production pose a microbial food safety risk .In the sixth month of storage, the counts of total plate and total coliform for sample 1 (which contains 28% MTSF) were the lowest compared with other samples, whereas the counts of yeast and mold for sample 2 (which contains 14% MTSF) and sample 3 (which contains 28% WF) were the lowest compared with sample 1. The demand from consumers for plant-based meat alternatives is rising quickly, but it is still challenging for food processors to produce high-quality goods in the necessary quantities. As a result, products must be further developed while taking food safety considerations into account.

6.2 .Chemical composition:

The present study reveals that wheat flour replacement with milk thistle seed flour (MTSF) shows a significant difference in the composition of soy plant-based meat analogues. Overall, the result indicated the high content of protein fat, fiber, and ash in the soy protein based meat analogue formulated with 28% MTSF. Furthermore, the MTSF showed lower values in moisture and carbohydrate than either wheat flour . In conclusion, MTSF is suitable for the formulation of soy protein based meat analogues because it already contains a considerable amount of protein, fiber, lipids, and minerals that have nutritional and functional properties and are minimally present in a wheat flour. Moreover, due to the high content of flavonolignans, summarily called silymarin, and their positive effects on the liver, it is also suitable for the production of functional foods

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6.3. Peroxide value:

Peroxide value (PV) generally increased significantly over the course of storage. Additionally, after being frozen for six months, the peroxide value (PV) of the soy-based meat analogue made with milk thistle seed flour reduced ($P < 0.05$) as the percentage of milk thistle seed flour increased.

6.4. TBARs:

The results indicated that the TBA values increased throughout the six months of storage. In addition, the TBAR's value after four months of storage started to slow down in soy-based meat analogues formulated with 28% MTSF samples compared to other meat analogue samples. This indicates that the utilization of MTSF in meat analog samples is able to slow down the oxidation rate. In conclusion, lipid oxidation occurred in all soy plant-based meat analogue samples, but at a low rate. In addition, the longer the meat analogues were stored in the freezer, the more lipids were oxidized.

6.5 Sensory evaluation:

The outcomes of the current study stated that the sensory evaluation highlighted an intense odor and taste profile of the soy protein-based meat analogue formulated with 28% MTSF, whereas the soy plant-based meat analogue formulated with 14% MTSF had more neutral sensory characteristics. Additionally, the textural properties of the soy plant-based meat analogue that contains 28% milk thistle seed flour had higher hardness and chewiness compared to the soy plant-based meat analogue that contains 28% and 15% wheat flour. Further research is needed to modulate and calibrate each of the sensory attributes for specific purposes and target consumers during the formulation of the meat analogue.

6.6. Recommendations:

- According to research, adding milk thistle to soy plant-based meat substitutes may improve their qualitative traits and result in a healthier end product.

- To establish the right proportion of milk thistle seed flour to utilize in soy plant-based meat alternatives, more research is required.
- The sensory evaluations of milk thistle seed flour for usage in plant-based meat substitutes also require additional study.
- Effects of milk thistle seed flour on the physicochemical properties of meat analogues: more research is required.
- I recommend investigating the potential for milk thistle cultivation in Palestinian territory.

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تأثير دقيق بذور الخرفيش على الخواص الكيميائية والميكروبية والحسية للحوم النباتية المحتوية على بروتين الصويا

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الملخص

هدفت هذه الدراسة لمعرفة تأثير استخدام دقيق بذور الخرفيش على الخصائص الميكروبية والكيميائية والحسية لبدائل اللحوم المصنعة من بروتين الصويا. أظهرت النتائج في الفحص الأول لتعداد البكتيري أن العينة التي تحتوي على 28٪ دقيق بذور الخرفيش لها تعداد بكتيري عال (1±8 CFU/g) مقارنة بعينات اللحوم النباتية الأخرى ولكن بعد مرور 6 أشهر من تجميدها لوحظ أن هنالك انخفاض كبير في التعداد الميكروبي لذات العينة. أما تعداد العفن والفطريات فقد كان مرتفع في العينة التي تحتوي على 14% من دقيق بذور الخرفيش (5.3 CFU/g) في الفحص الأول. أيضا لوحظ أن جميع العينات ملوثة بالبكتيريا القولونية، والتي تراوح تعدادها (2 - 7.67 CFU/g) خلال 6 أشهر من التجميد. بشكل عام، انخفضت جميع التعدادات الميكروبية بشكل ملحوظ كلما زادت فترة التخزين. كذلك أظهرت النتائج أن بدائل اللحوم التي تحتوي على دقيق بذور الخرفيش لها نسب بروتين ومعادن ودهون وألياف عالية، ونسب كربوهيدرات ورطوبة منخفضة مقارنة ببدائل اللحوم التي تحتوي على دقيق القمح .كذلك خلال فترة التخزين، هناك انخفاض غير ملحوظ في محتوى البروتين والدهون والكربوهيدرات والألياف والرماد لجميع العينات ولكن العينة التي تحتوي على 28% من دقيق بذور الخرفيش لوحظ أن هنالك زيادة ملحوظة في المحتوى الرطوبة بنسبة 0.43٪ بينما كان هناك انخفاض في محتوى الرطوبة في كل من العينات التي تحتوي 0% و14% من دقيق بذور الخرفيش. بعد ستة أشهر من التخزين كانت أعلى قيمة بيروكسيد (PV) في العينة المحتوية على 0٪ من دقيق بذور الخرفيش كذلك زادت قيمة بيروكسيد (PV) لهذه العينة (0.65 mEq/kg - 1.70) خلال الستة أشهر من التخزين. وأيضا تم ملاحظة أعلى قيمة لـ TBARsفي عينات اللحوم النباتية التي تحتوي على 0 ٪ من دقيق بذور الخرفيش أ. زادت قيم TBARs لهذه العينة من 0.21 mg/kg في أول فحص إلى 0.27 mg/kg بعد ستة أشهر من التخزين. كما أظهرت النتائج أن هناك زيادة ملحوظة في قيم البيروكسيد (PV) و TBARSكلما زادت فترة التخزين. أما في التقييم الحسي للعينات قيم أعضاء اللجنة أن بدائل اللحوم النباتية المصنعة من بروتين الصويا التي تحتوي على 0٪ و14٪ من دقيق بذور الخرفيش على أنها تمتلك خصائص حسية أفضل من تلك التي تحتوي على 28٪ من دقيق بذور الخرفيش.