FORMULATION, CHARACTERIZATION, AND SHELF LIFE STUDY OF GLUTEN-FREE COOKIES USING HILL GRAINS OF NEPAL

A Dissertation submitted to the Department of Microbiology,
St. Xavier's College (Affiliated to Tribhuvan University),
Kathmandu, Nepal, in Partial Fulfillment of the Requirements for
the Award of Degree of Master of Science in Microbiology
(**Food and Industrial)**

by:
DURGA PATHAK
T.U Symbol No: MB1383/075
T.U Regd No: 5-2-33-117-2010
2023

RECOMMENDATION

This is to certify that **Ms. Durga Pathak** has completed her dissertation work entitled **"Formulation, Characterization, and Shelf Life Study of Gluten-Free Cookies Using Hill Grains of Nepal"**, as partial fulfillment of requirements of the Master of Science Degree in Microbiology (**Food and Industrial Microbiology**) under my supervision. To my knowledge, this work has not been submitted for any other degree.

……………………….

Mr. Prakash Manandhar
Lecturer
Department of Microbiology
St. Xavier's College
Maitighar, Kathmandu, Nepal

……………………

Dr. Laxaman Sherchand
Director
Nepal Dairy and Modern food
Pvt. Ltd
Dhapakhel, lalitpur , Nepal

Date: ………………

CERTIFICATE OF APPROVAL

On the recommendation of **Mr. Prakash Manandhar (Lecturer**), Department of Microbiology, St. Xavier's College, this dissertation work of **Ms. Durga Pathak** entitled **"Formulation, Characterization, and Shelf Life Study of Gluten-Free Cookies Using Hill Grains of Nepal"**, has been approved for the examination and is submitted to the Tribhuvan University in partial fulfillment of requirements of Master's degree in Microbiology **(Food and Industrial Microbiology)**.

……………………….

Ms. Pramila Parajuli
Head
Department of Microbiology
St. Xavier's College
Maitighar, Kathmandu, Nepal

Date: …………………..

BOARD OF EXAMINERS

Recommended by:

……………………………

Mr. Prakash Manandhar

Supervisor

…………………………….

Dr. Laxman Sherchand

Supervisor

Approved by:

………………………………

Ms. Pramila Parajuli

Head

Department of Microbiology

St.Xavier's College

Examined by:

………………………………

Mr. Sandesh Maharjan

Lecturer

St.Xavier's College

Internal Examiner

………………………………..

Prof. Dr. Binod Lekhak

Tribhuvan University

Central Department of Microbiology

External Examiner

Date: ……………………………..

ACKNOWLEDGEMENTS

I would like to express my sincere gratitude to my respected supervisors **Mr. Prakash Manandhar**, Lecturer, Department of Microbiology, St. Xavier's College, Kathmandu, and **Dr. Laxman Sherchand**, Director of Nepal dairy and Bakery food industry, for their continuous support and expert guidance throughout my research work.

I am very delighted and want to express my thankfulness to my Head of Department **Ms. Pramila Parajuli** for providing me a workable environment to accomplish this research work and for constant encouragement throughout the dissertation. Similarly, I would like to share my sincere gratitude to **Dr. Heramba Bahadur Rajbhandary** (Executive chairman of Nepal Dairy Pvt. Ltd.) **Mr. Sunil Rajbhandary** and **Mrs. Anjula Rajbhandary** (Director of Modern Foods) for providing me research grant and work space to conduct my research in the Modern Food Department.

I am indebted to **Ms. Asmita Pandey** (Lab in charge of Nepal dairy), **Mr. Gopi Neupane,** and **Mrs. Anju lama** for their great efforts and cooperation during laboratory work.

Without the guidance of the entire staff, the members of the Microbiology Department, and the members of St. Xavier's family, this piece of work would not have been achievable. I thus express my sincere gratitude to them.

Finally, I would like to thank all the members of Nepal Dairy, my family members, and my friends for their encouragement and equivalent help.

…………………

Ms. Durga Pathak

Date: ………………

ABSTRACT

The consumption of bakery items made from refined flour has increased in the past decade, resulting in a rapid rise of gluten-related disorders. The purpose of this study was to develop, characterize, and study the shelf life of various gluten-free cookies (GFC) using indigenous hill grain flours of Nepal, fungal α-amylase, baking powder, and baker's yeast as leaveners. Various composite flours were prepared by millet, maize, buckwheat, and taichin rice flour in 75:25%, baked at 200°C for 20 minutes, proofed at 30°C until 1 hour for food grade fungal α-amylase with improver and baker’s yeast treated sample before mixing with cream. The moisture content of all formulated cookies was below 5% except BWBY sample during the 60 days of storage period. BWBY cookies showed highest moisture content, ranging from 6 % to 8.37% during 60 days of storage. The ash content of cookies samples were found to be significantly increased from 0.81% to 2.59%. Cookies made with 75:25 % millet-taichin rice flour blend treated with food grade fungal α-amylase with improver and baker’s yeast, received the highest scores for taste (8.0), texture (7.6) and crunchiness (8.1) out of 9 point headonic scale. Gluten-free cookies (GFC) were found to have a higher ash and moisture content compared to traditional cookies. The bacterial and fungal growth was not observed on both PCA and PDA plates at 0 days of storage. The microbial load and moisture content increased over time in most of the cookie samples. However, the microbial counts remained within acceptable levels (below 1×10^4cfu/gm) and the moisture content below 5% throughout the entire storage. Despite a marginal decrease, the overall acceptability (OAC) score of the all cookies was still well accepted (7 out of 9) by panelists after 60 days of storage. Based on their nutritional, sensory, and microbial qualities, the cookies made with a combination of millet-taichin rice flour (MABK), and maize-taichin flour (MzABK) were found to be good in quality until the end of the storage period (60 days).

Key words: Gluten free cookies, Millet-rice flour cookies, Maize-rice flour cookies, Buckwheat-rice flour Cookies, Whole wheat grain cookies

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF PHOTOGRAPHS

LIST OF APPENDICES

ABBREVIATIONS

ALT	Total Plate Number /Mesophilic Aerobic bacteria Test
AOAC	Association of Official Analytical Chemists
BWBK	Buckwheat-taichin flours cookies using baking powder
BWBY	Buckwheat-taichin flours cookies using Baker’s yeast
BWABK	Buckwheat-taichin flours cookies using baking powder and amylase enzyme
BWABY	Buckwheat-taichin flours cookies using Baker’s yeast and amylase enzyme
CFU/ml	Colony Forming Unit per ml
D/W	Distilled Water
D.F	Dilution Factor
FAO	Food and Agriculture Organization of the United Nations
GRF	Glutinous Rice flour
MBK	Millet-taichin flours cookies using baking powder
MBY	Millet-taichin flours cookies using baker’s yeast
MABK	Millet–taichin flours cookies using baking powder and amylase enzyme
MABY	Millet-taichin flours cookies using baker’s yeast and amylase enzyme
MzBK	Maize-taichin flours cookies using baking powder
MzBY	Maize-taichin flours cookies using baker’s yeast
MzABK	Maize-taichin flours cookies using baking powder and amylase enzyme

MzABY	Maize-taichin flours cookies using baker's yeast and amylase enzyme
OAA	Overall Acceptability score
PCA	Plate Count Agar
PDA	Potato Dextrose Agar
SMP	Skim Milk Powder
TPC	Total Plate Count
WBK	Whole grain wheat–taichin flours cooking using baking powder
WBY	Whole grain wheat–taichin flours cooking using baker's yeast
WABK	Whole grain wheat–taichin flours cookies using baking powder and amylase enzyme
WABY	Whole grain wheat-taichin flours cookies using baker's yeast and amylase enzyme
Wt.	Weight
W/V	Weight/Volume
WHO	World Health Organization
YMC	Yeast and Mold Count

CHAPTER I

INTRODUCTION

1.1. Background

Cookies are a widely consumed baked goods item because they are culturally accepted, safe to eat, reasonably priced, and ready to eat. They are made from a dough that is made from cereals, sweeteners, shortenings, and leavening agents and are transformed into a desirable product through baking. Wheat flour, shortening, sugar, salt, and water are the main ingredients for making cookies, which are then combined with additional minor components such as baking powder, skimmed milk, ammonium bicarbonate, and sodium bicarbonate to create a gluten-rich dough. Cookies primarily consist of two unhealthy ingredients: refined wheat flour and saturated fat .As public apprehension regarding gluten intolerances increases, the demand for gluten-free substitutions has risen markedly (Martínez et al 2022) .

Consumers are now more cognizant of the value of eating whole grains that are gluten-free and high in protein and fiber. As a result, researchers have conducted numerous studies to examine the use of blended flours (derived from grains, pseudocereals, and root crops) as a substitute for wheat flour in developing functional products like cookies. Despite the benefits of these blended flours, achieving the desired physical and sensory properties of the resulting products remains a challenge. Nevertheless, by blending a variety of grains like millet, rice, maize, buckwheat, quinoa, etc., researchers were able to create cookies that are both satisfying in terms of their physical and sensory qualities (Chopra et al 2018).

Functional foods are a modern dietary trend that not only supply needed nutrients but also help avoid disease. This trend is endorsed by global nutrition organizations as a response to various health problems associated with wheat ingestion, including celiac disease, diabetes, and coronary heart disease (WHO/FAO 2003). As a result of this situation, there is a growing demand for low-carbohydrate diets, slowly digestible starchy foods, and an increased consumption of functional foods (Hurs and Martin 2005). Therefore, this research work is designed to produce different hill grain-based cookies to improve the nutritional quality by developing low gluten cookies and increasing the utilization of locally available cheap grain flours in the hilly region of Nepal. As the prevalence of metabolic disorders, obesity, and gluten sensitivity rises, there is a growing desire to create fortified products that are rich in dietary fibers (DF) and possess antioxidant (AO) properties. Additionally, these products should have a reduced gluten content while still maintaining a pleasant taste (Zlatanović et al 2019).

Gluten is a viscoelastic protein found in wheat, rye, and barley that aids dough rise and adds flavor. As a result of these factors, it is widely used in a variety of food products (including non-cereal), resulting in an increase in the overall quantity of gluten consumed in a typical Western diet (Weiss 2021). Gluten-related disorders(GRD) have arisen as a significant phenomena that could have an impact on health-care procedures and expenses around the world (Schiepatti et al 2020). Gluten-related diseases (GRD) encompass a wide range of symptoms that are all triggered by the consumption of gluten (Sapone et al 2012). The number of people suffering from gluten-related disorders (GRDs) continues to grow, with a global estimate of 5% of the population affected. Taraghikhah et al (2020) stated that there are five significant types of gluten-related disorders (GRDs), which are celiac disease (CD), dermatitis herpetiformis (DH), gluten ataxia (GA), wheat allergy (WA), and non-celiac gluten sensitivity (NCGS). These disorders can manifest with a broad spectrum of symptoms. Celiac disease (CD), dermatitis herpetiformis (DH), and gluten ataxia (GA) are classified as autoimmune diseases, while wheat allergy (WA) and non-celiac gluten sensitivity (NCGS) are categorized as allergic and non-autoimmune-allergic diseases, respectively (Sapone et al 2012; Rostami et al 2008; Sharma et al 2020).

Less than 20 ppm of gluten is one prerequisite for food products to be marked "gluten-free". Gluten-free cookies are baked with gluten-free flour or low-gluten components that have a gluten content of less than 20 parts per million (ppm). According to some researchers studying celiac disease and some epidemiological data, most people with the condition are able to tolerate a range of traces and concentrations of gluten in food, even amounts less than 20 parts per million (ppm), without experiencing any negative health effects (US FDA 2020). In the current study, rice (*Oryza sativa*), maize (*Zea mays*), sorghum (*Sorghum vulgare*), and pearl millet (*Pennisetum glaucum*) flours were used to replace traditional wheat (*Triticum aestivum*) flour in the production of gluten-free cookies (Rai et al 2014).

Leavening agents play various roles in baked products. The capability of leavening agents to give baked products a high volume and aerated structure is the most important activity, but not the only one (De Leyn 2014).
Sodium bicarbonate, often known as baking soda ($NaHCO_3$), decomposes into sodium carbonate, water, and carbon dioxide when heated. Carbon dioxide is less water soluble and thus exits as a gas. Sodium carbonate (Na_2CO_3) decomposes in an acidic atmosphere, producing carbon dioxide. Sodium bicarbonate has certain drawbacks. Only around 25% of the sodium bicarbonate dissolves, leaving an alkaline environment that causes chemical degradation. This chemical breakdown manifests itself as a yellowish crumb and surface color, as well as a disagreeable flavor, particularly at larger doses (Manley 2000).

At temperatures above 60°C, ammonium bicarbonate (NH_4HCO_3) decomposes quickly. Ammonia gas (NH_3), water, and carbon dioxide are released during decomposition. Ammonia and carbon dioxide exit as gases, leaving no residue behind in the baked food. However, if the formula contains a lot of moisture, ammonia will dissolve in the water phase and change the flavor. The low final moisture content of ammonium bicarbonate assures minimum retention of dissolved ammonia. Because ammonium bicarbonate decomposes quickly when heated, it has a rising action and spreads less, resulting in a coarse structure and large volume (De Leyn 2014).

Baking powder is a combination of ingredients that functions as a leavening agent. It is made of sodium bicarbonate, one or more acidic components, and a filler, such as starch or calcium carbonate (Pop 2007). Baking powder is classified into two types: single-acting and double-acting. Single-acting baking powder includes only one acid, which can react quickly during mixing or slowly in the oven. On the other hand, double-acting baking powder has both fast-acting and slow-acting acids, with the majority of carbon dioxide release happening in the oven. The ideal double-acting baking powder will produce 20% carbon dioxide during mixing and the remaining in the oven (Brodie 2006).

The use of sourdough has become increasingly popular in enhancing the taste, structure, and shelf life of baked goods. Fermenting cereal products also has the potential to enhance the nutritional value and health benefits of foods and ingredients. Sourdough not only improves the taste of whole grain, fiber-rich, or gluten-free products, but it can also slow down the digestion of starch, leading to a lower glycemic response. Furthermore, sourdough fermentation can modify the levels and accessibility of beneficial compounds and improve the absorption of minerals. Poutanen et al (2009) suggest that the fermentation of cereals can lead to the production of non-digestible polysaccharides or alter the accessibility of the fiber in the grain to the gut microbiota. They also propose that the breakdown of gluten in bread during fermentation may enhance its suitability for individuals with celiac disease.

The main component of wheat flour, maize, millet, taichin rice and buckwheat flour is starch. Amylases are enzymes that break down starch into small dextrins and oligosaccharides, which yeast can use during dough fermentation, proofing, and early baking stages. This results in an improved volume and texture of baked goods. The small sugars and oligosaccharides produced by amylases also enhance the Maillard reaction, which is responsible for the crust's browning and appealing flavor. Fungal α-amylases are a type of endoacting amylase that breaks down alpha - 1,4 glycosidic bonds found in the inner part (endo) of amylose or amylopectin chains in starch polymers. These calcium metalloenzymes are unable to function without calcium and break down long-chain carbohydrates at random locations, ultimately producing maltrotriose and maltose from amylose, or maltose, glucose, and limited dextrin from amylopectin (Tiwari et al 2015).

The consumption of refined wheat flour (maida) found in baked goods like cookies and muffins can lead to Gluten-related disorders (GRDs). Conventional cookies made with refined wheat flour are deficient in dietary fiber, vitamins, minerals, and are high in carbohydrates and unsaturated fat. To address this issue, cookie recipes have been modified by substituting bleached wheat flour with whole grain millet flour, maize flour, buckwheat flour, wheat flour, and taichin rice flour. These flours are rich in protein, total dietary fiber, vitamins, minerals, and antioxidants. This substitution is believed to help prevent celiac disease, obesity, diabetes, and gastritis. Thus, there is a need for standard operating procedures to formulate, characterize, and study the shelf life of gluten-free cookies made from locally available and underutilized hill grains in Nepal. Although cookies are a staple in many diets, from army personnel to hospitalized individuals, limited studies have been conducted on gluten-free cookies that entirely replace wheat flour with other flour blends. Therefore, this research aims to create, analyze, and determine the shelf life of gluten-free cookies that are entirely free of wheat flour to address the problem of malnutrition and gluten-related diseases caused by consuming baked goods made with refined wheat flour.

1.2. Objectives

1.2.1 General Objective

To develop, characterize, and study the shelf life of various gluten-free cookies using different hill grain flours.

1.2.2 Specific Objectives

1. To develop various gluten-free cookies using different hill grain flours, food grade fungal α-amylase enzyme with improver, and leavening agent (baker's yeast, baking powder).
2. To assess the microbial quality of formulated cookies.
3. To study the physical and chemical properties of different cookies
4. To evaluate sensory characteristics of prepared cookies
5. To determine the shelf life of gluten-free cookies

CHAPTER II

LITERATURE REVIEW

2.1 Cookies

As worldwide travel gained popularity during that era, cookies became a convenient snack to bring along on trips, replacing the traditional travel cakes that were used in the past. The jumble, a firm cookie made primarily from flour, nuts, sweeteners, and water, had a hard texture that made it durable for travel was one of the earliest and most popular cookies that traveled well and became famous across continents with similar names (Hossain et al 2021).

One of the earliest sorts of cuisine was cookies. Because they are a type of dried food, there is a never-ending demand for cookies. They are even supplied to areas affected by natural disasters or in remote locations and isolated community. In recent times, new types of cookies have been developed by altering their ingredients. Bakery products, including cookies, have played a significant role in human civilization. One of the advantages of cookies is that they provide a quick and easy source of nutrient-rich food. Additionally, in the modern world, cookies are often enjoyed with tea, and the two have become strongly associated with each other (Gandhi et al 2001).

2.2 Principle component of traditional and gluten free cookies

The main component in conventional cookies is refined wheat flour, which is a highly concentrated source of starch and contributes to around 80% of the total energy in the flour. The cookies also contain elevated levels of fat and sugar, but only minimal amounts of fiber. This lack of fiber increases the risk of various health issues, such as constipation, diabetes, obesity, cancer, high blood cholesterol, and coronary heart disease. As a result, these cookies are not considered a well-balanced diet, which conflicts with the values of health-conscious consumers today. To meet the needs of these consumers, fortifying the cookies with natural ingredients appears to be the only solution that can enhance both the aesthetic and nutritional quality of the product, according to Awolu et al (2016). Cookies are typically made with basic ingredients such as wheat flour, fat (usually shortening or butter), sugar, and water, but other ingredients like milk, salt, emulsifier, aerating agent, flavor, and color can also be added. These ingredients can be supplemented with other nutrients to meet specific dietary or therapeutic requirements. The main flour used in baking, including for cookies, is usually wheat or composite flour. However, many flours used in bakery products are bleached, which has been linked to negative health effects from long-term consumption. Some researchers refer to refined flours as "slow poison" or "glue of the gut" and discourage consumption or recommend reducing intake. On the other hand, recent studies have shown that consuming whole wheat and grain-based products can reduce the risk of chronic diseases and age-related disorders, such as cardiovascular diseases, type II diabetes, obesity, and even some types of cancer. Most commercially available biscuits and cookies are made with bleached flour (maid), but using whole grain flour made from whole kernel grains can provide a range of health benefits due to the presence of antioxidants,vitamins,minerals and dietary fiber. Bleaching flour/Refined wheat flour can result in a loss of minerals and vitamins and can produce alloxan, which is used to induce diabetes in rats (Peter Ikechukwu et al 2017).

Rao et al (2018) concluded that wheat flour is the principal component of virtually all cookies; however, good quality products can be prepared using non-wheat (non-gluten) flours, such as xiquexique (*Pilosocereus gounellei*) flour and other gluten-free cereals grain. Wheat flour can be substituted with other flours, such as buckwheat flour,rice flor and millet flour, made from fruits, leaves,gluten free grains, tubers, and vegetables (Granato and Ellendersen 2009).

In comparison to refined wheat flour, whole grain wheat flour is considered to include a notable quantity of macronutrients, such as carbs, lipids, and proteins; micronutrients, such as vitamins and minerals; phytochemicals, TDF, and numerous bioactive components. Carotenoids and anthocyanins, which are natural colors, can be found in colored wheat (Ficco et al 2016). B-group vitamins B1 (thiamine), B2 (riboflavin), B3 (niacin), B6 (pyridoxine), B9 (folate), and vitamin E (tocopherol/tocotrienol) are all abundant in yellow colored wheat. In addition to these, a trace amount of provitamin A (bcarotene), vitamin D (calciferol), and vitamin K (phylloquinone) are also present (Balyan et al 2013).

2.3 Wheat-Gluten:

Gluten, which is a protein present in wheat flour, plays a significant role in giving wheat the unique ability to produce leavened baked goods. It can be described as the cohesive, visco-elastic protein material formed as a by-product of starch isolation from wheat flour. Vital wheat gluten has distinct visco-elastic properties that enhance dough strength, mixing tolerance, and handling characteristics. Its ability to form films provides gas retention and controlled expansion for enhanced volume, consistency, and texture. Its thermosetting characteristics contribute to necessary structural rigidity and bite attributes, and its water absorption capacity enhances yield, softness, and shelf life of baked goods (Day et al 2006).

Vital wheat gluten refers to a wheat protein product that exhibits high viscoelasticity when hydrated. Its viscoelastic properties primarily depend on the interaction between two types of storage proteins found in wheat: gliadins and glutenins. Gliadins exist predominantly as individual monomeric structures, whereas glutenins form polymeric structures interconnected by disulfide bonds. Glutenins, with their ability to form intermolecular disulfide bonds, can cross-link and create a network of gluten, which enhances the elasticity and strength of dough. On the other hand, the presence of gliadins weakens the gluten network by incorporating themselves into its structure, resulting in increased viscosity. Achieving a proper balance between gliadins and glutenins is important to achieve optimal baking performance. Apart from the gliadin/glutenin ratio, the manufacturing process itself also impacts the quality of vital gluten (Schopf et al 2021). During wheat-based food processing, the gluten protein network is typically developed and established. Heat-induced aggregation of gluten occurs through cross-linking within and between its protein fractions. Prominent reactions involved in this process include the oxidation of sulfhydryl (-SH) groups and SH- disulfide (SS) bonds interchange, leading to the formation of SS cross-links. Additionally, other covalent bonds are also formed (Delcour et al 2012).

2.4 Gluten-free food

Gluten is a structure-forming protein that is required for the formulation of high-quality cereal-based products, so replacing it is the most difficult technological challenge. The only appropriate cure for gluten-related diseases is to follow a gluten-free diet for the rest of one's life (Gallagher et al 2004; Altındağ et al 2014). Khairuddin and Lasekan (2021) reported that gluten-free food products are initially made for consumers who suffer from gluten-related diseases such as wheat allergies, gluten ataxia, non-celiac gluten sensitivity, wheat-dependent exercise-induced anaphylaxis (WDEIA), and the most well-known, celiac disease. Gluten-free products are made up of gluten-free cereals /grains or raw materials.

Cereals that are free from gluten or contain less than 20 parts per million of gluten fall under the category of gluten-free cereals (Khoury et al 2018). A gluten-free diet permits the consumption of foods made from rice, corn, soy, pea, millet, potato, sorghum, amaranth, quinoa, and buckwheat flours and their derivatives (Schuppan et al 2005).

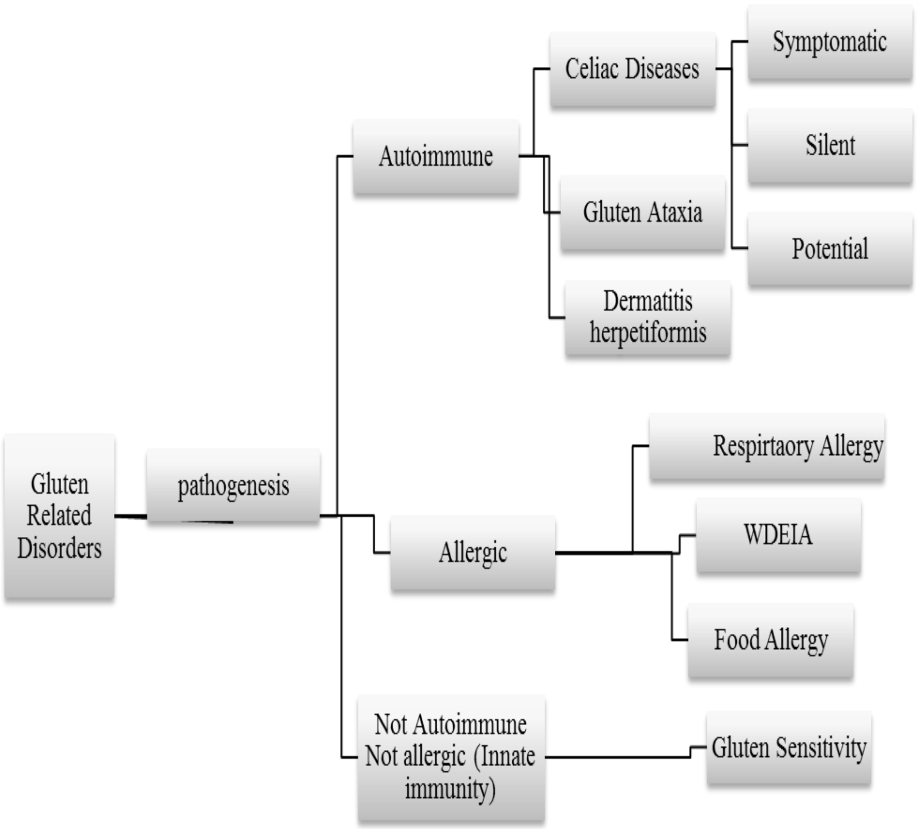
2.5 Gluten related disorders (GRDs)

Over the years, the consumption of gluten has been associated with various clinical conditions. Gluten-related disorders (GRDs) have become increasingly recognized as a significant epidemiological issue, with an approximate global prevalence of 5%. Celiac disease, wheat allergy, and non-celiac gluten sensitivity are distinct gluten-related disorders (Elli et al 2015).

Celiac disease (CD) is a persistent autoimmune disorder in which celiac patients' immune systems respond to gluten, which is made up of prolamin proteins found in wheat, rye, and barley. Abdominal distension, anorexia, chronic or recurrent diarrhea, failure to thrive or weight loss, vomiting, muscle atrophy, celiac crisis (rare), and exhaustion are the hallmark symptoms of the condition (Catassi and Fasano 2008).

Non-Celiac Gluten Sensitivity (NCGS) was initially identified in the 1980s and is a syndrome that has recently been "rediscovered." This condition is defined by intestinal and extra-intestinal symptoms that arise from consuming gluten-containing foods in individuals who do not have celiac disease (CD) or wheat allergy (WA), as reported by Catassi et al (2013).

The ground-breaking effort of Sapone and associates was to thoroughly analyze the spectrum of gluten-related illnesses and suggest new nomenclatures to address the gaps in present classifications (see Figure 1).



Sourced by: Sapone et al (2012)

Figure 1 Classification of gluten-related disorders (GRDs)

2.6 Nutritional value of Gluten-free hill grains of Nepal:

The Nutritive value of Nepalese cereals grains distribution in the hilly region of Nepal is given in the Table no.1. For each of the cereals listed, include values for energy, nutrients (such as protein, fat, vitamins, and minerals), as well as other significant food components (such as fiber). These estimates are based on chemical analysis done in the DFTQC's (Department of Food Technology and Quality Control) analytical laboratories (Karn 2017).

Table 1: Nutritive value of Nepalese cereals grains distribution in the hilly region of Nepal

Cereal grain	Carbohydrates	Minerals	Protein	Moisture	Fat	Fiber
Buck wheat flour	69.2gm	3.1gm	6.1gm	12.2gm	1.3gm	7.8gm
Finger millet	72gm	2.7gm	7.3gm	13.1gm	1.3gm	3.6gm
Maize flour	72.1gm	1.2gm	9.2gm	12gm	3.9gm	1.6gm
Wheat flour	69.4gm	2.7gm	12.1gm	12.2gm	1.7gm	1.9gm
Taichin rice flour	77.9gm	1.08gm	7.68gm	10.89gm	1.94gm	0.56gm

*Nutrients per 100 Grams

Source: Adapted from Food Composition Table (DFTQC 2017)

2.7 Formulated various gluten-free cookies

Composite flours refer to blends of various flours that are derived from grains, legumes, or other plant sources, either with or without wheat flours. The increased nutritional value and improved digestibility of composite flours have made them increasingly popular. Additionally, the use of composite flours can help mitigate the shortage of wheat production in many tropical countries and promote the use of unconventional domestic resources in flour production, reducing waste. The use of composite flours has been particularly emphasized in the preparation of baked goods, as they provide a valuable source of high-quality proteins, essential amino acids, vitamins, minerals, and dietary fibers (Chakraborty et al 2021). A variety of composite flours have been successfully used in making different types of cookies (Cheng and Bhat 2016; Zouari et al 2016).

2.7.1 Buckwheat (*Fagopyrum esculentum*) based cookies:

Buckwheat is a widely consumed staple food grain in Nepal, ranking 6th after rice, wheat, maize, finger millet, and barley. It is typically grown in hills at high altitudes over 1700 meters as a summer crop, while in mid-hills (600-

1700 meters above sea level) it is grown as an autumn or spring crop, and as a winter crop in the terai region, as explained by Luitel et al (2017). Common buckwheat or sweet buckwheat (*Fagopyrum esculentum*) and Tartary buckwheat (*Fagopyrum tartaricum)* are the two most commonly cultivated species of buckwheat in Nepal (Baljeet et al 2010). Buckwheat is devoid of cholesterol and has a significant nutritional value because of its balanced amino acids and minerals. Buckwheat grains are rich in essential vitamins such as thiamine (vitamin B1), riboflavin (vitamin B2), and pyridoxine (vitamin B6), while its protein content has a well-balanced amino acid composition with a high biological value (kato et al 2001). Additionally, buckwheat is gluten-free and a significant source of microelements such as zinc, copper, manganese, and selenium, as well as macroelements including potassium, sodium, calcium, and magnesium (Stibilj et al 2004; Wei et al 2003). Due to its therapeutic value, buckwheat is in high demand, particularly in urban areas.

Alvarez-jubete et al (2010) discussed the addition of pseudo-cereals to gluten-free products, and buckwheat flour was examined as an ingredient in gluten-free bread and cookies. Rice flour and buckwheat flour were used to make gluten-free cookies, and it was found that the cookies made with a mixture of the two flours had a significantly higher total phenolic content and antioxidant activity compared to control rice cookies. Cookies enriched with 30% buckwheat malt expressed a high content of protein, fiber, and resistant starch and a lower caloric value compared to RC (Rice) and TFC (Tartary buckwheat flour cookies), as reported by Torbica et al (2012) and Molinari et al (2017). The cookies were prepared according to the formulation described by Torbica et al (2012), where mixtures of rice and buckwheat flour were made in ratios of 90:10, 80:20, and 70:30, while rice flour was used in the preparation of control cookies.

Using buckwheat flour in composite flour-based cookies is a viable option due to its beneficial nutraceutical properties and gluten-free nature, which can be significant in preventing celiac disease. Cereal grains, including soft wheat, have lower protein content (ranging from 7 to 14%) and are deficient in some essential amino acids such as lysine. However, buckwheat has a superior protein quality than other cereals and can be used to supplement specific amino acids like lysine, histidine, valine, and leucine (Baljeet et al 2010 and Chopra et al 2014).

Chopra et al (2014) conducted a study to investigate the impact of adding buckwheat flour to cookies on their physical, chemical, and sensory properties. The study concluded that buckwheat flour can be effectively incorporated into wheat flour up to a level of 75% to create cookies with improved nutritional value and acceptable sensory characteristics. Consequently, the development and utilization of such functional foods can not only enhance the overall nutritional status of the population but also benefit those who have degenerative illnesses.

The buckwheat-wheat flour cookies, containing 75% buckwheat flour, 25% wheat flour, and an egg, had a moisture content of 15.76% and an ash content of 1.64%. Increasing the wheat flour concentration to 100% resulted in an improvement in the top grain, appearance, and texture scores, which reached 8.75. The overall score of wheat cookies was higher compared to buckwheat cookies. However, increasing the buckwheat flour concentration to 100% led to a reduction in the top grain, appearance, texture, and flavor scores to 5.25. This was due to the formation of cracks caused by gluten-free buckwheat flour. The flavor score was significantly reduced to 5.71 at higher concentrations, which may be attributed to the presence of flavonoid compounds, such as rutin, that have a bitter taste in buckwheat flour. The cookies made with a combination of 75% buckwheat flour and 100% wheat flour received high overall acceptability scores (Chopra et al 2014). .

2.7.2 Finger Millet (*Elensine coracan*) based cookies

Finger millet, (*Eleusine coracana*) is an annual grain that is grown primarily in Nepal, commonly known as Kodo, and is also popular in India, Philippines, Indonesia, Vietnam, Thailand, and in West Africa from where it originated. In terms of area and production, finger millet (Kodo) is Nepal's fourth most important crop after rice, maize, and wheat. Finger millet, a subsistence food crop, is a unique cereal with high nutritional quality particularly in hilly regions in Nepal (Luitel et al 2020). Millets consist of slow-releasing carbohydrates and thus lower the risk of diabetes. Also found to be rich in dietary fibers, minerals like iron, magnesium, phosphorous, and potassium (Michaelraj and Shanmugam 2013). Millets are a suitable dietary option for individuals with celiac disease due to their lack of gluten protein (Thompson 2009). Millet flour is increasingly being used in baked goods, particularly in gluten-free and diabetic-friendly bread, cookies, and crackers (Rai et al 2014 and Tanwar and Dhillon 2017). Refined wheat flour (maida) is widely used in bakery products such as cookies, and replacing it with finger millet flour will improve the nutritional quality of these products (Sinha and Sharma 2017).

As the level of Pearl Millet Flour (PMF) in cookies increased, it was observed that the moisture, ash, fat, and crude fiber content increased, while the protein and carbohydrate content decreased. The higher moisture content in the cookies may be due to PMF having a greater capacity for retaining moisture than maida, as reported by Rathi et al (2004). Additionally, the significant increase in ash content in cookies supplemented with PMF may be attributed to its higher PMF content compared to maida. Rathi et al (2004) reported comparable findings for pearl millet biscuits, which had a higher ash content (1.75%) and fiber content (7.8%) compared to the control sample (0.82% and 1.96%, respectively). Similarly, Kulthe et al (2018) observed an analogous pattern of increased moisture content (4.68%) and ash content (1.69%) in pearl millet cookies when compared to the control sample, which had a moisture content of 2.32% and an ash content of 0.86%.

Swami et al (2013) conducted a study where cookies were made entirely from finger millet flour and were baked using various temperatures (180, 200, and 220°C) and durations, along with different oil content (30, 35, and 40%), while maintaining consistent amounts of other ingredients such as baking powder, sugar, and vanilla flavor. The sensory evaluation results demonstrated that the texture, color, and overall quality of the cookies were rated higher (7 out of 9) on the 9-point hedonic scale for the cookies prepared with finger millet flour that had 30% oil content and were baked at 200°C for 20 minutes, as opposed to cookies made with different oil content and baking temperatures.

Sinha and Sharma (2017) conducted a study to create nutritious cookies using finger millet and subsequently evaluated their sensory and nutritional characteristics. Two variants of cookies were prepared by substituting 30% and 50% of refined flour with finger millet. Both types of cookies were found acceptable in terms of sensory evaluation. The incorporation of finger millet flour led to an increase in the overall mineral, fiber, calcium, iron, phosphorus, copper, and zinc content of the cookies. For the creation of nutrient-dense cookies, finger millet flour can be successfully substituted up to 50%.Sensory panelists did not prefer cookies made from blends containing over 50% millet flour. However, the phenolic content was higher in cookies made with either water washed or alkali washed millet flour compared to those made with plain wheat flour, with a value of 1.90 ± 0.14 mg gallic acid/g sample (Hussain et al 2020).

2.7.3 Maize or corn (*Zea mays*) based cookies:

Maize (*Zea mays* L.) is a crucial cereal crop and widely consumed due to its high nutritional value, containing substantial amounts of macronutrients such as starch, fiber, protein, and fat, as well as micronutrients including vitamin B complex, ß-carotene, and essential minerals like magnesium, zinc, phosphorus, and copper. Additionally, maize is a rich source of antioxidants that help safeguard against various degenerative ailments, as reported by Bathla et al (2019). According to Kataria (2014), incorporating maize-based products into the regular diet of individuals with coeliac disease can enhance their overall health. Celiac disease is a condition in which gluten cannot be properly absorbed due to an abnormal immune response that takes place in the small intestine. The only known remedy for this condition is the consumption of a gluten-free diet, which helps to improve gastrointestinal function, as observed by Gallagher et al (2004).

Whole Grain Maize Flour is created by grinding whole maize grains into flour. The addition of maize bran to various food products provides additional fiber content, while maize germ is highly nutritious and contains essential oils and proteins necessary for the human body. However, products that incorporate maize bran tend to be too sweet, which has prompted a reduction in sugar in the recipe. This can have health benefits for consumers by reducing sugar intake. Muyanja et al (2020) found that the inclusion of maize germ in baked goods not only enhanced their visual appeal but also improved their nutritional value. By incorporating maize germ and bran or using whole grain maize flour, it is feasible to produce high-fiber baked products that are both acceptable and of high quality. Gluten-free cookies were created using rice flakes, maize, and soybean flour, and their quality was evaluated. The resulting cookies, which contained soybean and maize flour, were recommended for individuals with gluten intolerance and could serve as a source of protein for those with protein deficiencies. Additionally, the study found that the cookies were microbiologically safe when compared to commercially available whole wheat flour cookies (Abiramy et al 2017).

Akbar and Ayub (2018) conducted a study where they used a mixture of common wheat and maize flour to prepare cookies in seven different ratios: F0 (100% wheat flour), F1 (90% wheat flour + 10% maize flour), F2 (80% wheat flour + 20% maize flour), F3 (70% wheat flour + 30% maize flour), F4 (60% wheat flour + 40% maize flour), F5 (50% wheat flour + 50% maize flour), and F6 (100% maize flour). The cookies made with the F2 (80% wheat, 20% maize flour) formulation were found to have the best overall results. The study suggests that further research is needed to explore the use of different flavors to enhance cookie acceptability, as well as employing specific packaging materials for cookie storage.

2.8 Taichin Rice (glutinous rice flour /Sticky rice flour) :

Rice flour made from both non-glutinous and glutinous rice is high in easily digestible carbohydrates and free of gluten and low in sodium. As a result, it has gained popularity as a substitute ingredient in gluten-free baked goods including cakes, breads, and biscuits, as well as in other food products such as pasta, noodles, and baby foods (Jiamjariyatam et al 2022). Sticky rice or waxy rice, also known as glutinous rice (*Oryza sativa*), is primarily made up of amylopectin and contains very little amylose. When compared to non-glutinous rice, cooked glutinous rice grains stick together, resulting in a unique, flexible texture. This is because the viscous properties of gelatinized starch are mainly due to amylopectin. The branched amylopectin structure in cooked glutinous rice grains has less tendency to retrograde, resulting in a slower rate of retrogradation than amylose (Qiu et al 2021) .

Taichin rice (*Oryza sativa* subsp. *japonica*) is variety of glutinous rice , is being used in cereals ,chips,snacks, and coating applications to provide different textures. Gluten-free flours that are commonly used for baking include glutinous rice flour (sticky rice flour) and various types of rice flours. To achieve the best dough and baked product quality, these flours are often combined with flours, starches, and proteins from cereals, pulses, pseudocereals, and other plant materials. While the composition of these composite flours and their formulations play a significant role in determining the texture and taste of the final product, other factors like the milling process, particle size of the flour, and flour treatment may also affect the outcome (Xu et al 2020)

2.9 Leavening agents

A substance that can cause doughs and batters to expand by releasing gases (CO_2), leading to the formation of porous baked goods, is known as a leavening agent. Unlike yeast fermentation, leavening agents use a chemical process to produce CO_2. Leavening agents are also referred to as raising agents.

Baker's Yeast: Leavening can be achieved through the process of fermentation, a biological process where microorganisms such as yeast or bacteria metabolize organic matter in food. The most commonly used strain of yeast in the bakery industry is *Saccharomyces cerevisiae*. Yeast releases zymase to ferment sugars, producing ethanol and CO_2. During baking, most of the alcohol evaporates, and CO_2 provides leavening. This process gives a distinct aroma and flavor to the final product (Neeharika 2020).Baker's yeast can be found in various forms, with the main distinction being the amount of moisture present. Dry yeast forms are ideal for long-term storage without experiencing a substantial decline in viability. Typically, the yeast flavor is not detectable in the baked bread if the amount of yeast added is less than 2.5% of the baker's total ingredients (Linda and Stanley 2007). The bread dough's quality was greatly improved by fermenting with lactic acid bacteria (LAB) derived from an overnight culture to achieve a concentration of about 5.0E+07 cells per gram or by adding baker's yeast (1.25%). The use of biological leavening with baker's yeast or sourdough fermentation has a significant impact on the texture and quality of gluten-free baked goods (Javaria et al 2016).

Baking powder: Baking powder is a comprehensive leavening system that offers a one-stop solution. It is made up of sodium bicarbonate, one or more leavening acids, and a diluent, such as starch or calcium carbonate. The acid and salt components react with each other when they come into contact in the dough's liquid phase. To prevent an immediate reaction, they are kept apart by an inert component, such as dry starch. Baking powders can be categorized as single-acting or double-acting based on their release type. Single-acting baking powders have a single acid that can either react quickly during mixing or slowly during baking. In contrast, double-acting baking powders contain both fast-acting and slow-acting acids. A double-acting baking powder is considered optimal when it releases a small quantity of carbon dioxide (around 20%) during the mixing process and the rest of it during the baking process (Otero-Guzmán et al 2020).

2.10 Fungal Alpha-Amylase (FAA) as cookies improver

The hydrolytic enzymes that break down starch, namely α-amylase, β-amylase, and glucoamylase, are among the most commonly utilized enzymes in modern biotechnology and food industry. Glucoamylases, also called amyloglucosidases, are a type of exo-acting amylase that catalyzes the production of glucose from starch and its corresponding oligosaccharides' non-reducing end. Glucoamylases are commercially employed for the conversion of malto-oligosaccharides into glucose. Bacteria and fungi usually secrete amylases outside their cells to digest starch extracellularly into sugars. *Bacillus* species, such as *B. amyloliquefaciens*, *B. subtilis*, *B. licheniformis*, *B. stearothermophilus*, *B. megaterium*, *B. circulans* and *Enterobacter* species have been extensively used in the industry to produce α-amylase (Luang-In et al 2019). Various fungi such as *Aspergillus oryzae*, *Aspergillus niger*, *Aspergillus kawachii*, *Aspergillus awamori*, *Penicillium roquefortii*, *Penicillium chrysogenum*, and *Streptomyces rimosus* have been studied as sources for producing α-amylase through submerged and solid-state fermentation (SSF). SSF is regarded as a favorable technique for the industrial-scale manufacturing of enzymes, according to Sundarram and Murthy (2014).

The addition of alpha-amylase as a flour ingredient improves the bread volume, enhances the flavor development, and acts as an anti-staling agent (Gujral et al 2003; Ananingsih et al 2012). Alpha-amylase, classified as a glycohydrolase (GH), is an enzyme that hydrolyzes α-1,4 and α-1,6 linkages in a random manner. Its main function is to break down starch into dextrins. However, it can only act on starches that are either damaged or gelatinized, as these forms make them susceptible to enzymatic degradation (Oort 2010; Ananingsih et al 2012). However, extensive degradation of damaged starch as a result of high alpha-amylase level will lead to sticky dough. Therefore, an appropriate dosage of alpha-amylase is necessary to be able to improve the quality of dough and final product.

Incorporating α-amylase into dough leads to an acceleration in the fermentation process and a decrease in dough viscosity, which subsequently improves the texture and volume of the resulting product. Additionally, the enzyme produces more sugar within the dough, enhancing the taste, crust color, and toasting properties of baked goods.The shelf life of baked goods is prolonged by the inclusion of α-amylases, which also help to maintain freshness and improve the softness of baked goods (Mobini-Dehkordi and Javan 2012).

2.11 Proofer and its mechanism

A proofer, also referred to as a proving oven or proofing cabinet, is an oven that maintains a temperature range of 95-110°F (35-43°C) and a relative humidity of 80-85% to stimulate yeast activity in fermenting dough and α-amylase activity. Proofing is a vital step in bread making, along with kneading, as it contributes to the development of the bread's aroma and creates an aerated structure in the dough. During kneading, air comprising oxygen and nitrogen gets trapped inside the dough, forming bubbles. During kneading, yeast (*Saccharomyces cerevisiae*) activity starts, utilizing almost all of the trapped oxygen, and it continues during leavening. The enzymes hydrolyze sugars from starch, which are then consumed by the yeast to produce ethanol and carbon dioxide. As carbon dioxide has low bubble-forming ability and high solubility, it diffuses into the gas nuclei formed during kneading and dissolves in the aqueous phase. Nitrogen is mainly responsible for gas nuclei formation since oxygen is almost entirely consumed by the yeast. Most of the carbon dioxide diffuses into the gas nuclei and causes them to expand. While saturation is not mandatory for CO_2 diffusion into air nuclei, it typically takes place once the solution is saturated by carbon dioxide (Chiotellis and Campbell 2003 and Gally et al 2017)

2.12 Microbiology of gluten free cookies

Bacterial and YMC growth were found to be prevalent in unprocessed buckwheat flour, whereas processed wheat flour did not exhibit any microbial growth. However, when buckwheat flour was used to make cookies and processed at a high temperature (200°C for 12 min), no microbial growth was observed in the analysis conducted by Chopra et al (2014). Buckwheat flour has APC (aerobic plate count) and YMC (yeast and mold count) restrictions set at 5.5 and 4.7 $\log_{10}$ colony forming units (CFU)/g, respectively, by some consumers. It was found that a lower dosage of acidic calcium sulfate (ACS) might accomplish sufficient microbial reduction and lessen the effect on the product's color. According to Dhillon et al (2012), the most efficient treatment for ACS (acidic calcium sulfate) was at a concentration of 50 mL/L, which led to a 3.9 $\log_{10}$ decrease in APC and the total eradication of YMC.

Banusha and Vasantharuba (2014) discovered that fresh samples of both Wheat flour biscuit (WB) and Composite biscuit (CB) did not show any bacterial or fungal colonies. After a month of storage, the bacterial count was 6.8×10^2 CFU/g for WB and 2.3×10^2 CFU/g for CB. After two months, the bacterial count increased to 9.6×10^2 CFU/g for WB and 3.6×10^2 CFU/g for CB. However, no fungal growth was observed during the two-month storage period. The acceptable bacterial count level for biscuits is below 1×10^4 CFU/g, and both WB and CB samples met this safety standard for up to two months of storage.

Over a period of 4 weeks, the number of microbes in the millet flour-based cookies decreased by 2.05×10^2, 1.05×10^2, and 4.0×10^1 at temperatures of 25°C, 37°C, and 44°C. Despite this decrease, the cookies still meet the Indonesian National Standard for cookie quality (SNI standards. 01-2973:2011), which specifies a maximum of 1×10^4 colonies/gram.The decrease in microbial contamination in the ALT test is thought to be caused by the limited oxygen available in the packaging and the stability of the storage temperature. In the microbial test in ALT it is referred to as aerobic microbial count (AMC) or aerobic plate count (APC), meaning that these microbes require free oxygen to grow and develop. Lack of oxygen in the packaging causes ALT microbes cannot grow (Anggraini and salam 2021).

2.13 Shelf life of cookie

The mathematical model of shelf life states that, as the storage temperature increases, the shelf life decreases because higher temperatures lead to faster reaction rates that cause the cookies to spoil more quickly, resulting in a shorter shelf life. The chemical reactions occur at a quicker rate at higher temperatures, which leads to a faster decline in product quality. The durability of millet flour cookies varies depending on the storage temperature, with respective shelf lives of 134.81 days (4.5 months), 79.18 days (2.6 months), and 59.14 days (1.9 months) when stored at temperatures of 25°C, 37°C, and 44°C, respectively. The shelf life of a food item is heavily influenced by its water content, as a lower water content inhibits the growth of microbes.

Conversely, a higher water content in food speeds up the damage caused by microbial activity, since the microbes require free water to grow (Anggraini and salam 2021).

In a study by Sakač et al (2016), it was discovered that sensory properties may be a more reliable predictor of shelf-life for gluten-free rice-buckwheat cookies than the measurement of total aldehydes content. Based on the analysis of sensory characteristics, it was found that the most significant degradation in sensory quality of rice-buckwheat cookies was caused by an increase in hardness, a decrease in fracturability, and the appearance of unusual odors and flavors. It was determined that color development was not a reliable indicator of sensory changes during the 6 months storage period of the cookies. Both unpacked and packed cookies maintained satisfactory sensory scores for the mentioned properties for up to 3 months and 5.5 months, respectively, when stored at room temperature (23 ± 1°C).

CHAPTER III

MATERIALS AND METHODS

3.1 Materials

All the materials, types of equipment, reagents, and media used during the study are listed in Appendix A.

3.2 Methodology

3.2.1 Study Site

The study was carried out in the Food Microbiology laboratory, St. Xavier's College, Maitighar, Kathmandu, Nepal, and the Modern Food and Bakery department of Nepal Dairy Private Limited (nd's), Dhapakhel, Lalitpur, Nepal.

3.2.2 Duration of study

The study was conducted from 9th January 2022 to 15th June 2022.

3.2.3 Study design

Standard microbiological protocols were followed for the collection as well as the processing of the sample. GHP (Good Hygienic Practice) and GMP **(**Good Manufacturing Practice) were adopted during preparing and processing of the product. This research work was a laboratory-based qualitative study.

3.2.4 Sample size and sample types

- A total of 16 samples of gluten-free cookies were formulated, characterized, and shelf life studied during the study period.
- **Shelf life study:** at 0 day, 30 days, and 60 days from the packaging date of cookies

3.3 Collection of Raw materials

3.3.1 Raw materials: Maize flour, Millet flour, Wheat flour, Buckwheat flour, and Taichin rice flour (glutinous rice flour) were procured from Shiva Mill Center in Lokanthali, Bhaktapur, Nepal.

Baker's yeast, Baking powder, icing sugar, skim milk powder, butter, salt, and custard powder were obtained from the Bakery department of Nepal Dairy Pvt Ltd (nd's), Dhapakhel, Lalitpur, Nepal.

Food grade fungal α-amylase (FAA) enzyme with improver was taken from the food Microbiology lab of St. Xavier's College, Maitighar, Kathmandu, Nepal

3.3.2 Control sample: For the control sample, Refined wheat flour (maida) cookies manufactured by Nebico Private limited was taken.

3.3.3 Raw materials processing

The high-quality hill grains were cleaned, grading, milling, and sieving to produce high-grade grain flours.

Maize flour, Millet flour, Wheat flour, Buckwheat flour, and Taichin rice flour (glutinous rice flour) were sieved for the separation of coarse particles from finer particles.

3.4 Sample formulation

3.4.1 Formulation of different gluten free cookies

Gluten free cookie was developed by modifying the method 10-50.05 (American Association of Cereal Chemists, 2010) and utilized the findings from Brites et al (2019). All 16 different cookies sample were prepared according to the following protocol and recipe.

For the preparation of cookies 3 major steps were followed:

1. Preparation of composite flours

2. Making the whipped cream

3. Preparation of the dough

1. Preparation of composite flours

Composite flours were prepared by slightly modifying given procedure in (Chopra et al 2014).For the buckwheat-taichin (glutinous) rice composite flour,75% i.e 375 gm well cleaned and sieved buckwheat flour was mixed with 25% i.e 125 gm taichin rice flour for 500 gm composite flours. A similar proportion was added to each sample.

For the preparation of buckwheat-taichin rice flour cookies, 125 gm taichin rice flour was mashed up with pre-weighed hot water to create a sticky texture then added 375 gm buckwheat flour and mixed it well. For the production of BWBK, BWABK, BWBY, and BWABY cookies, baking powder, baking powder + amylase enzyme, baker's yeast, baker's yeast + amylase enzyme was added as leavening agent respectively. After the addition of leavening agent except for baking powder only and enzyme, composite flours were kept in proofer at 30°C for 1 hour to activate the growth of yeast and enhance enzymatic activity. Detailed descriptions of preparing composite flours are given in the flow chart (Chopra et al 2014).

2. Making the whipped cream

Well whipped cream was developed by slightly modifying the procedure as described by Kulkarni et al (2021). All the given ingredients were weighed accurately according to the below table. The pre-weighed icing sugar, shortening, butter, custard powder, sodium bicarbonate, ammonium bicarbonate, skim milk powder (SMP), butter flavor, and salt in specific proportion were mixed thoroughly. Then mashed up manually until dough-like formation. After that egg shaker was used to mix up all ingredients and produced white well-whipped cream. At last, water was added and blended by egg shaker for 2-5 minutes until a creamy white color was developed. The recipe for cream formation is given in table no 2.

Table 2: Recipe for the cream formation

S.N	Ingredients	Weight in gram
1.	Icing sugar	200 gm (40%)
2.	Shortening	150 gm (30%)
3.	Butter	80 gm (16%)
4.	Sodium Bicarbonate	2.5 gm (0.5%)
5.	Ammonium Bicarbonate	2.5 gm (0.5%)
6.	Custard powder	10 gm (2%)
7.	Skim milk powder	20 gm (4%)
8.	Salt	5 gm (1%)
9.	Butter Flavor	4 gm (0.8%)
10.	Water	120 gm (24%)

3. Preparation of the dough and production of cookies

For dough preparation the method used by Singh et al (2008) was followed with some modification. The kneading process was used to prepare the dough. The dough was prepared by mixing the composite flours in well-blended cream. Then the well-formed dough was filled up in a nozzle piping bag and cut the dough according to the desired shape and size with the help of the nozzle and baked in the pre-heated digital Rotating Rack Convection Oven at 200°C for 20 minutes, after baking the cookies were allowed to cool for 15 minutes at room temperature and then sealed in an air tight plastic container with plastic before further analysis.

Raw ingredient pre-weighed (clean and well sieved 375gm millet flour +125 gm taichin flour (glutinous rice flour) for 500gm)

preparation of composite flours

At first, mix the glutinous rice flour in 30 gm hot water

↓

Mashed up manually until became a sticky texture

↓

Then added 375 gm millet flour and mixed up properly

↓

Added 40 gm water and mixed thoroughly for 5 minutes

↓

MBK cookies → 4gm of baking powder was added

MBY cookies → 10.41gm baker's yeast was added

MABK cookies → 0.1 gm fungal amylase enzyme + 4gm of baking powder was added

MABY cookies → 0.1 gm fungal amylase enzyme +10.41gm baker's yeast was added

↓

All of composite flours were kept in proofer at 30°C for an hour to encourage yeast growth and increase enzymatic activity after the addition of leavening agents and enzymes, except MBK coded composite flour in which only baking powder was added as leavening agent.

↓

Composite flours are ready to mixed up with well whipped creame

Cream was prepared by adding all ingredients given in a table no.2

↓

Preparation of dough: mixed up composite flours with well-formed cream

↓

Cutting the dough using nozzle piping bag to give appropriate shape

↓

Baked at 200°C for 20 minutes in preheated Rotating Rack Convection Oven (digital)

↓

Cooled down at room temperature for 15-20 minutes after baked

↓

Kept in a plastic container and packaged with a polypropylene (PP) plastic bag

↓

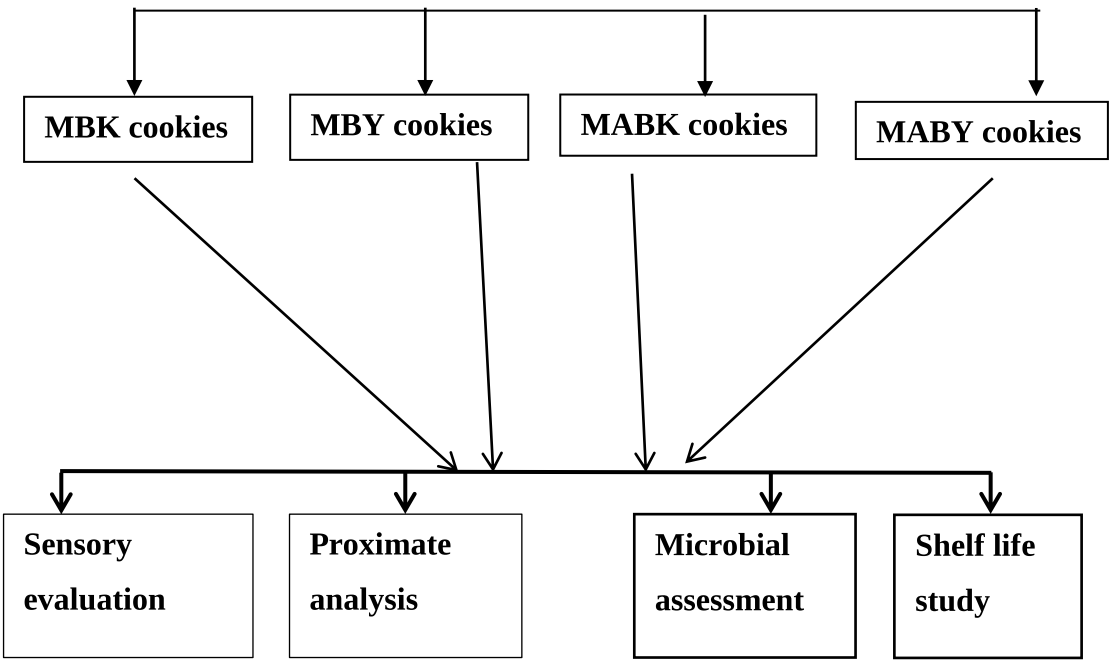


Figure 2: Flowchart showing the formulation process of Millet-glutinous rice flour (3:1) cookies and its laboratory analysis

3.5 Laboratory analysis

The formulated cookies were stored in PET box and packaged with a polypropylene (PP) plastic bag in a room temperature from where samples were drawn for laboratory analysis (Galić et al 2009).

3.5.1 Microbial assessment of developed cookies

In 16 different formulated gluten-free cookies, total plate count (TPC) and yeast and mold count (YMC) were used to measure the viable load of microorganisms. The serial dilution pour plate method and the spread plate method were used to determine it. The number of colonies on dilution plates was counted, averaged, and reported as cells per gram (cfu/gm) (Aneja 2007).

$$\text{Colony forming unit /gram (CFU/gm)} = \frac{\text{No. of colonies (Mean)} \times \text{D.F}}{\text{Volume of sample used 1ml or (0.1 ml)}}$$

3.5.2 Proximate Analysis

The proximate value (moisture content, ash content, and qualitative test of carbohydrate) of cookie samples was determined.

Table 7: Method used for proximate analysis

Test	Method
Moisture %	By Hot air oven
Ash %	By muffle furnace
Carbohydrates	By qualitative tests

1.Moisture content of cookies samples

Moisture content of cookies samples was determined by the Hot air oven method. In this method, formulated cookie sample was weighed and heated in an insulated oven to constant weight. The difference in weight is the water that has evaporated.The oven should be automatically controlled, with the temperature regularly set at 100°C or 105°C. A 5 gram sample was placed in an aluminum moisture disc and heated to 105°C for 4 hours (Rai and K.C 2007). After that moisture content (%) was calculated using the following formula:

$$\text{Moisture Content (\%)} = \frac{W1-W2}{W} \times 100\%$$

Where,

W = Wt. of sample

W1= Wt. of aluminum moisture disc +sample before oven drying

W2 = Wt. of aluminum moisture disc +sample after hot air oven drying at 105°C for 4 hours

2. Ash content of cookies samples

Ash content of cookies samples was determined by the Dry Ashing method. In this method, ash percentage was determined by AOAC (1990) method. About 2-5gm of each sample was ignited at 560°C in a muffle furnace for 6 hours. The residue was cooled in a desiccator and weighed. Total ash content was determined using the following formula:

$$\text{Percentage of Ash (dry basis)} = \frac{\text{Mass of ash (MASH)}}{\text{Mass of sample}} \times 100\%$$

Another formula was also used ,described by Ranganna(2012):

$$\text{Ash (\%)} = \frac{W1-W2}{W} \times 100\%$$

Where, W1 = wt. of crucible +ash

W2 = wt. of crucible

W = wt. of sample

3. Qualitative tests for Carbohydrates:

1. **Molisch's test:**

   In this test, 2ml of different cookie sample solutions were taken in different dry test tubes and labeled it well. As a control, 2ml of distilled water was added in another tube. 2-3drops of molisch's reagent were added to the sample solution.1-2ml of conc.H_2SO_4 was added along the side of the tube. Reddish-violet-colored products were developed at the interface of two compounds (Shrestha 2002).

2. **Iodine's test**

   2ml of different test samples were taken in different dry test tubes and labeled it well. 2ml of distilled water was taken in another tube as control. 2-3drops of iodine solution were added to each test sample tube. The blue-dark color was developed in each test sample. An iodine test was done for the identification of starch in the cookie sample (Shrestha 2002).

3. **Seliwanoff's test**

   For this test, 1ml of cookies sample was taken in a test tube.5ml of Seliwanoff's reagent was added to each sample test tube. Then, heated to boil for 30 seconds, the formation of red color in the test sample indicates the presence of ketoses sugar (Shrestha 2002).

4. **Fehling's test:**

   1ml of different cookies sample solutions were taken in different dry test tubes and labeled it well. 1ml of freshly prepared Fehling's reagent was added to each tube. After that, the tubes were kept in a boiling water bath and then observed for red precipitates. The presence of red precipitate indicates that the test was positive (Shrestha 2002).

5. **Bial's test**

   Bial's test was performed to detect the presence of pentoses and pentosans (derivatives of pentoses) in cookie samples. 2ml of bial's reagent was taken in different tubes and labeled it well. 4-5 drops of test solution were added to each reagent tube. Kept in the water bath for boiling. The development of green color was observed on each tube (Shrestha 2002).

6. **Barfoed's test:**

For this test, 2ml of different sample's solution was taken in a clean and dry test tube. 2ml of barfoed's reagent was added to it. Then the tubes were kept in a boiling water bath and noted the time until brick red precipitation was observed (Shrestha 2002).

- Development of brick red precipitate within 3-5 minutes indicates reducing monosaccharaides.
- Development of brick red precipitate within 7-12 minutes indicates reducing disaccharides.

3.5.3 Sensory Analysis of prepared cookies:

Sensory evaluation of the cookies samples was carried out by a team of sixteen inexperienced (16) panelists. The panelists were instructed to evaluate the coded samples based on the acceptability of their color, flavor, texture, and overall acceptability by a 9-point hedonic scale. Each sensory attribute was carried out, with panelists adopting the multiple comparison test system. The average score was calculated from individual organoleptic properties.

A panel of judges evaluated the quality of prepared cookies in the sensory evaluation. A hedonic rating test with a 9-point scale from "like extremely" to "dislike extremely" was used to measure (Chopra et al 2014).

Hedonic Rating Scale

9. Like Extremely	6 . Like Slightly	3. Dislike Moderately
8. Like Very much	5. Neither Like nor Dislike	2. Dislike Very Much
7. Like Moderately	4. DislikeSlightly	1. Dislike Extremely

3.5.4 Shelf life study

The shelf life studies of 16 different cookies were conducted for 60 days in PET box packaging material and sealed with Polypropylene (PP) plastic bag for the consideration of various parameters such as Overall Acceptability (OAA) score, moisture content, and microbial quality of cookie at dry storage condition and room temperature. The inspection and observation took place within 24 hours (0 day) of the manufactured date, 1 month (30 days), and 2 months (60 days) later, respectively (Wandhekar et al 2021)**.**

3.6 Data Management and Analysis

To analyze the major findings, the raw data obtained from the laboratory experiment were tabulated and presented in defined tables. Statistical Package for the Social Sciences software version 23 (SPSS), Statistical Analysis System (SAS) version 6.07, and MS Excel was used to manage and analyze the raw data.

CHAPTER IV

RESULTS

4.1. Cookies prepared in different combinations

The gluten-free cookies were prepared by entirely substituting refined wheat flour (known as maida) with a combination of hill grain flour (buckwheat flour, maize flour, and millet flour) and glutinous rice flour (taichin flour) in a 3:1 ratio. The developed cookies were then analyzed to determine their physical, chemical, and sensory properties, and the formulation recipes are presented below.

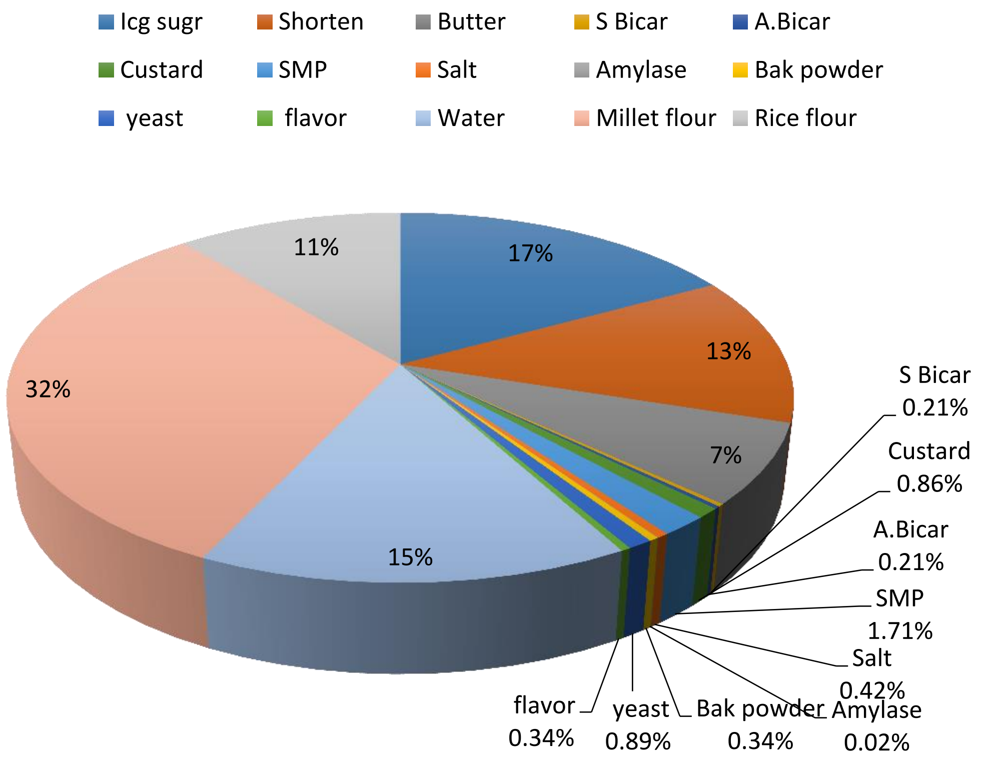


Figure 5: Formulation recipe of cookies

The above chart illustrates the formulation of gluten-free cookies using various ingredients in different proportions. It was found that composite flour, made up of millet flour and sticky rice flour, made up approximately 43% of the total ingredients. This means that the mixture contained 75% hill grains and 25% taichin rice flour in a total composite flour of 500 grams Additionally, 17% icing sugar was added as a sweetening agent, which was almost half the amount of composite flour used. Shortening and butter were added at the rate of 13% and 7% respectively. To ensure proper baking, 0.2% sodium bicarbonate and 0.2% ammonium bicarbonate were used. About 2% skim milk powder was added to improve the taste and texture of the cookies. 0.89% baker's yeast and 0.84% custard powder was included to create a good dough. Finally, a very small amount (0.02%) of amylase enzyme was treated during the dough-making process.

4.2. Ash content of cookies

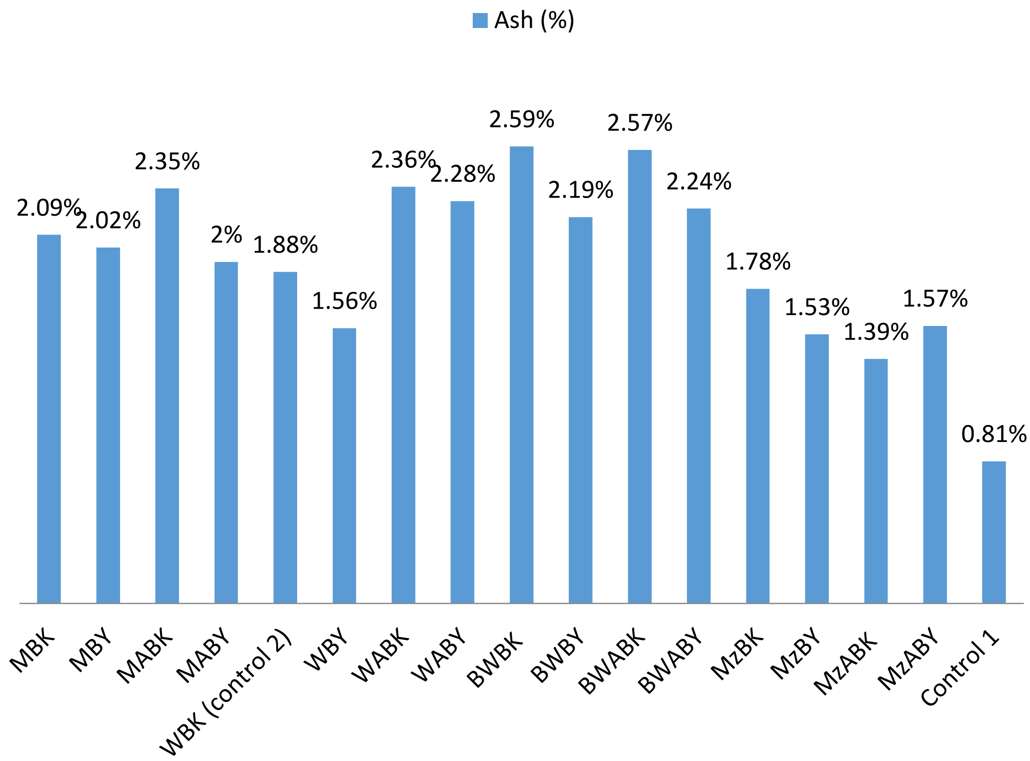


Figure 6: Ash content in formulated gluten free cookies and control

A column chart was used to display the data of ash content in cookies**.** Out of 16 cookie samples, sample BWBK contained the highest percentage of ash i.e 2.59% compared to the other samples. On the other hand, Sample MzABK contained the least quantity of ash, at 1.39% among the 16 cookie samples. When the test sample and control sample were compared, the control sample (C_1) had the lowest ash percentage i.e 0.81% of the total tested sample.

4.3 Moisture content of cookies

The moisture content of four different hill grain-based cookies was determined by the oven-drying method. The moisture content of 16 cookie samples was determined 3 times at 3 different periods.

Table 8: Moisture content of cookies

Sample	Moisture% at 0 day	Moisture% at 30 days	Moisture% at 60 days
MBK	1.15%	1.20%	2%
MBY	3.80%	4%	5.16%
MABK	1.37%	2.80%	4%
MABY	2%	2%	2.97%
WBK	1.57%	1.68%	2.80%
WBY	1.80%	2.06%	3.10%
WABK	2.20%	2.21%	3.20%
WABY	3%	3.08%	3.27%
BWBK	4.16%	4.40%	4.63%
BWBY	6%	6.83%	8.67%
BWABK	2%	3.49%	4.06%
BWABY	3%	3.37%	3.81%
MzBK	2.09%	2.38%	3.33%
MzBY	2.02%	2.09%	2.32%
MzABK	2.35%	2.50%	2.62%
MzABY	1.05%	1.94%	2.39%
Control	-	2.10%	-

Among the various cookie samples made from buckwheat flour, a buckwheat-based cookie with the code BWBY displayed the highest moisture content, at 6%, when compared to other samples within 24 hours of storage after baked and packaged . Other buckwheat-based cookies, such as BWBK, BWABK, and BWABY, had moisture contents ranging from 2% to 4.16%. On the other hand, the lowest moisture content of 1.05% was found in a maize-based cookie coded MzABY after 24 hours of storage. Other maize-based cookies, such as MzBK, MzBY, and MzABK, had moisture contents ranging from 2.02% to 2.35%.

After one month (30days) of storage, the buckwheat-based cookie with code BWBY had the highest moisture content of 6.83%, while the millet-based cookie with code MBY had the lowest moisture content at 1.20%. The control sample had a moisture content of 2.10%. Furthermore, cookies with codes MBK, MABY, WBK, WBY, MzBY, and MzABY had lower moisture content than the control sample.

After two months (60days) of storage , the cookie with code MBK had the lowest moisture content at 2%, while the buckwheat-based cookie with code BWBY had the highest moisture content of 8.67%, followed by BWBK and BWABK with 4.63% and 4.06%, respectively.

4.4 Microbiological evaluation of cookies

Within the initial 24-hour (0 days) of baked cookies, there was no detectable microbial growth on either Plate Count Agar (PCA) or Potato Dextrose Agar (PDA) plates.

Total viable count of developed cookies on the PCA after 60 days of storage at room temperature (25 ± 2°C):

The data in the table 9 represents the mean count of viable bacteria on PCA (plated count agar) of formulated gluten-free cookies at two different time intervals: on the 30th and 60th days after production and storage. The evaluation was performed by counting the total viable count on PCA (plate count agar). The units of viable count are given in colony forming units (CFU) per gram of cookie sample (cfu/gm). The MBK and MBY samples have the highest viable count among all the samples after 30 days (5.3×10^2 and 2.37×10^3 cfu/gm respectively). However, after 60 days, the MBY sample had the highest viable count (4.16×10^3 cfu/gm). The WABY, BWABY, and MzABY samples have the lowest viable count after both 30 and 60 days of storage. At 30 days after production, the mean count of viable bacteria in the control sample was 2.4×10^2 cfu/gm. This count was higher than the counts in several of the test samples, including WBK, WABK, BWBK, MzBK, and MzABK. However, the count was lower than the counts in several other test samples, including MBY, WBY, BWBY, MzBY, and BWABY.

Yeast and Mould Count (YMC) on PDA during the period of 60 days of storage:

The table 9 showed the yeast and mold count (YMC) in colony forming units per gram (cfu/gm) for each cookie sample at 30 and 60 days after production. After 30 days of storage, the cookies with codes MBK and MABK had highest YMC of 4.0×10^2 cfu/gm and 3.0×10^2 cfu/gm, respectively. The samples with the lowest YMC were MBY and MABY, at 2.0×10^2 cfu/gm. The remaining samples, including WBK, WBY, WABK, WABY, BWBK, BWBY, BWABK, BWABY, MzBK, MzBY, MzABK, and MzABY, showed no growth. The control sample had a YMC of 1.2×10^3cfu/gm.

After 60 days of storage, the samples with the highest YMC were MBK, MBY, and MABY, with 1.3×10^3 cfu/gm and 1.2×10^3 cfu/gm, respectively. The samples with the lowest YMC were MABK, WBK, WABY, BWBK, BWBY, BWABK, BWABY, and MzABY, each with a YMC of 2.0×10^2cfu/gm. All samples showed an increase in YMC over time. After 60 days of storage, the YMC of the sample with code MzABY was comparable to that of the control sample during the 30-day storage period.

Table 9: Mean count on PCA and PDA of formulated cookies after 0day, 30 days, and 60 days of storages

Sample	PCA (cfu/gm) 0day	PCA (cfu/gm) 30 days	PCA (cfu/gm) 60 days	PDA (cfu/gm) 0day	PDA (cfu/gm) 30 days	PDA (cfu/gm) 60 days
MBK	NG	5.3×10^{2}	3.01×10^{3}	NG	4.0×10^{2}	1.3×10^{3}
MBY	NG	2.37×10^{3}	4.16×10^{3}	NG	2.0×10^{2}	1.2×10^{3}
MABK	NG	1.8×10^{2}	2.6×10^{2}	NG	3.0×10^{2}	3.2×10^{2}
MABY	NG	1.8×10^{2}	2.1×10^{2}	NG	2.0×10^{2}	1.2×10^{3}
WBK	NG	6.0×10^{1}	2.6×10^{2}	NG	NG	2.0×10^{2}
WBY	NG	2.2×10^{2}	2.9×10^{2}	NG	NG	3.0×10^{2}
WABK	NG	1.6×10^{2}	1.8×10^{2}	NG	NG	3.0×10^{2}
WABY	NG	1.4×10^{2}	2.6×10^{2}	NG	NG	2.0×10^{2}
BWBK	NG	1.4×10^{2}	1.8×10^{2}	NG	NG	2.0×10^{2}
BWBY	NG	6.0×10^{1}	2.4×10^{2}	NG	NG	2.0×10^{2}
BWABK	NG	2.5×10^{2}	2.6×10^{2}	NG	NG	2.0×10^{2}
BWABY	NG	3.0×10^{2}	3.3×10^{2}	NG	NG	2.0×10^{2}
MzBK	NG	1.4×10^{2}	1.8×10^{2}	NG	NG	6.0×10^{2}
MzBY	NG	4.3×10^{2}	5.9×10^{2}	NG	NG	6.0×10^{2}
MzABK	NG	2.6×10^{2}	5.2×10^{2}	NG	NG	1.2×10^{3}
MzABY	NG	2.0×10^{2}	2.4×10^{2}	NG	NG	2.0×10^{2}
control	-	2.4×10^{2}	-	-	1.2×10^{3}	-

*NG indicates No growth of organisms

4.5 Sensory attributes of cookies

The sensory properties of formulated cookies were evaluated at three different times, i.e. 0 day, 30 days, and 60 days after production, to assess shelf life and determine the characteristics influencing customer preferences.

4.5.1 Sensory evaluation of cookies within 24 hours (0 day) of production:

Among the different types of cookies made with various flour combinations and leavening agents, those made with 75% whole grain wheat flour and 25% taichin rice flour and baking powder (BWK) had the highest overall acceptability rating, with a score of 8.25. However, cookies made with the same flour combination but using baker's yeast (WBY) as the leavening agent had the highest ratings in terms of color, texture, and flavor. Cookies made with 75% buckwheat flour and 25% taichin rice flour and baker's yeast (BWBY) had the lowest overall acceptability rating of 6.625. Despite this, all of the cookies were considered acceptable according to the hedonic scale, based on their appearance, color, texture, flavor, taste, and overall acceptability, except for the sample coded BWBY.

Table 10: Comparison of mean value on sensory attributes of gluten-free cookies after 0 day from packaging

S.N	Sample	Color	Appearance	Taste	Texture	Flavor	Crunchiness	Overall Acceptability
1	MBK	7.750^{abcd}	7.687^{bcd}	7.750^{abcd}	7.562^{bcd}	7.562^{bcde}	7.562^{bcde}	7.687^{cd}
2	MBY	7.625^{bcde}	7.062^{e}	7.062^{e}	7.062^{d}	7.375^{cde}	8.063^{ab}	7.562^{de}
3	MABK	7.500^{cde}	7.875^{abc}	7.812^{abcd}	7.500^{bcd}	7.500^{cde}	8.063^{ab}	7.625^{cde}
4	MABY	7.625^{bcde}	7.875^{abc}	8.000^{abc}	7.625^{bc}	7.562^{bcde}	8.125^{a}	7.875^{abcd}
5	WBK	8.000^{ab}	8.000^{abc}	8.250^{a}	7.750^{ab}	8.00^{ab}	8.188^{a}	8.250^{a}
6	WBY	8.188^{a}	8.125^{ab}	8.188^{ab}	8.188^{a}	8.063^{a}	8.063^{ab}	8.125^{ab}
7	WABK	8.188^{a}	8.000^{abc}	8.063^{abc}	7.875^{ab}	7.625^{abcd}	8.000^{abc}	8.000^{abc}
8	WABY	8.063^{ab}	8.000^{abc}	7.562^{cde}	7.500^{bcd}	7.437^{cde}	7.687^{abcde}	7.750^{bcd}
9	BWBK	7.375^{de}	7.375^{ed}	7.625^{bcde}	7.750^{ab}	7.312^{cde}	7.500^{cde}	7.562^{de}
10	BWBY	6.312^{f}	5.750^{f}	5.062^{f}	5.000^{e}	7.187^{de}	7.187^{e}	6.625^{f}
11	BWABK	7.187^{e}	7.312^{ed}	7.500^{cde}	7.437^{bcd}	7.437^{cde}	8.000^{abc}	7.625^{cde}
12	BWABY	7.875^{abc}	7.875^{abc}	7.625^{bcde}	7.687^{abc}	7.75^{abc}	8.063^{ab}	7.625^{cde}
13	MzBK	8.125^{a}	8.188^{a}	7.750^{abcd}	7.750^{ab}	7.562^{bcde}	7.937^{abc}	7.937^{abcd}
14	MzBY	8.000^{ab}	7.875^{abc}	7.625^{bcde}	7.625^{bc}	7.500^{cde}	7.375^{de}	7.750^{bcd}
15	MzABK	7.875^{abc}	7.625^{cd}	7.312^{de}	7.500^{bcd}	7.687^{abc}	7.812^{abcd}	7.562^{de}
16	MzABYY	7.187^{e}	7.125^{e}	7.125^{e}	7.187^{cd}	7.125^{e}	7.562^{bcde}	7.250^{e}

Mean with the same superscript letter within each column are not significantly different from each other at the 5% level ($P < 0.5$). N=256; 1-9 point hedonic scoring scale.9 Hedonic Rating Scale was used to measure on a scale of 9 points from "like extremely"

9. Like Extremely	6. Like Slightly	3. Dislike Moderately
8. Like Very much	5. Neither Like nor Dislike	2. Dislike Very Much
7. Like Moderately	4. Dislike Slightly	1. Dislike Extremely

4.5.2. Sensory evaluation of cookies after 30 days of production:

The given table compared the mean values of the sensory attributes of gluten-free cookies after 30 days from packaging ,with the control sample. As with the previous table, the letters after the mean values represent statistical significance, with mean values that have the same superscript letter with in each column being not significantly different from each other at the 5% level ($P < 0.5$). The scale of measurement for the data was the same 9-point hedonic rating scale, with higher values indicating higher levels of acceptability for the sensory attributes being measured. It showed that the samples WBK, WBY, WABK, and WABY had relatively high mean values for the sensory attributes of color, appearance, taste, texture, flavor, and crunchiness, as well as overall acceptability. On the other hand, the sample BWBY had relatively low mean values for these attributes.

The study found that several of the cookie samples, including MABK, MABY, WBK, WBY, WABK, WABY, BWABK, MzBK, and MzBY, received higher ratings for overall acceptability compared to the control sample, which had a score of 7.437 on the hedonic scale. This suggests that the sensory qualities of these gluten-free cookies were generally well-liked by participants, with many of the mean scores falling in the "like moderately" to "like very much" range. The control sample and the MBK sample, which was made with millet - taichin rice flour, had similar ratings for overall acceptability.

Table 11: Comparison of mean value on sensory attributes of gluten-free cookies after 30days from packaging

S.N	Sample	Color	Appearance	Taste	Texture	Flavor	Crunchiness	Overall Acceptability
1	MBK	7.5^{cdefg}	7.500^{abcd}	7.625^{abc}	7.437^{bc}	s7.437^{ab}	7.437^{bcd}	7.437^{cde}
2	MBY	7.437^{defg}	6.937^{e}	6.937^{d}	6.937^{c}	7.187^{b}	7.937^{abc}	7.375^{de}
3	MABK	7.375^{efg}	7.750^{abc}	7.687^{abc}	7.375^{bc}	7.250^{b}	7.937^{abc}	$7.500b^{cde}$
4	MABY	7.437^{defg}	7.750^{abc}	7.875^{ab}	7.500^{bc}	7.437^{ab}	8.000^{ab}	7.750^{abcd}
5	WBK	7.9375^{abcd}	8.000^{ab}	8.188^{a}	7.625^{ab}	7.937^{a}	8.063^{a}	8.188^{a}
6	WBY	8.188^{a}	8.063^{a}	8.188^{a}	8.188^{a}	7.937^{a}	7.937^{abc}	8.000^{ab}
7	WABK	8.188^{a}	7.750^{abc}	7.875^{ab}	7.625^{ab}	7.500^{ab}	8.000^{ab}	7.937^{abc}
8	WABY	8.000^{abc}	7.875^{ab}	7.437^{bcd}	7.375^{bc}	7.187^{b}	7.562^{abcd}	7.500^{bcde}
9	BWBK	7.250^{fg}	7.250^{bcde}	7.500^{bcd}	7.687^{ab}	7.187^{b}	7.375^{cd}	7.312^{de}
10	BWBY	6.312^{h}	5.750^{f}	5.062^{e}	5.000^{d}	7.187^{b}	7.187^{d}	6.312^{f}
11	BWABK	7.000^{g}	7.125^{de}	7.312^{bcd}	7.187^{bc}	7.375^{ab}	7.875^{abc}	7.375^{de}
12	BWABY	7.625^{bcdef}	7.562^{abcd}	7.437^{bcd}	7.375^{bc}	7.437^{ab}	7.937^{abc}	7.562^{bcde}
13	MzBK	8.063^{ab}	8.063^{a}	7.625^{abc}	7.625^{ab}	7.375^{ab}	7.750^{abcd}	7.750^{abcd}
14	MzBY	7.937^{abcd}	7.812^{abc}	7.500^{bcd}	7.562^{b}	7.375^{ab}	7.375^{cd}	7.687^{abcd}
15	MzABK	7.812^{abcde}	7.437^{bcde}	7.187^{cd}	7.375^{bc}	7.562^{ab}	7.750^{abcd}	7.375^{de}
16	MzABYY	7.000^{g}	7.000^{de}	6.875^{d}	7.187^{bc}	7.125^{b}	7.500^{abcd}	7.192^{e}
17	Control	7.812^{abcde}	7.562^{abcd}	$7.000d^{e}$	7.250^{bc}	7.000^{b}	7.375^{cd}	7.437^{cde}

Mean with the same superscript letter within each column are not significantly different from each other at the 5% level ($P < 0.5$). N=256; 1-9 point hedonic scoring scale.

9. Like Extremely	6. Like Slightly	3. Dislike Moderately
8. Like Very much	5. Neither Like nor Dislike	2. Dislike Very Much
7. Like Moderately	4. DislikeSlightly	1. Dislike Extremely

4.5.3 Sensory evaluation of cookies after 60days of production:

Based on the displayed data, it appears that the sensory attributes of the gluten-free cookies were rated highly by the panelists. Many of the mean values for the different samples fall within the "like moderately" to "like very much" range on the hedonic scale. This indicates that the panelists generally found the cookies to be acceptable and enjoyable.

After 60 days from production, the sensory characteristics of the cookies showed significant differences compared to those measured at 48 hours and 30 days. Most of the samples scored between 7 and 7.5, except for the MBY and BWBY-coded samples.

Table 12: Comparison of mean value on sensory attributes of gluten-free cookies after 60 days from packaging

S.N	Sample	Color	Appearance	Taste	Texture	Flavor	Crunchiness	Overall Acceptability
1	MBK	7.000^{cde}	7.437^{abcd}	7.250^{ab}	7.187^{a}	7.250^{a}	7.687^{abc}	7.187^{a}
2	MBY	5.812^{f}	6.312^{e}	6.125^{cd}	6.187^{bc}	6.125^{b}	6.312^{d}	6.187^{b}
3	MABK	7.125^{bcde}	7.187^{bcd}	7.250^{ab}	7.437^{a}	7.000^{a}	7.750^{ab}	7.437^{a}
4	MABY	7125^{bcde}	7.562^{abc}	7.125^{ab}	6.937^{a}	6.875^{a}	7.375^{abc}	7.125^{a}
5	WBK	7.562^{abc}	7.562^{abc}	7.625^{a}	7.250^{a}	7.312^{a}	7.250^{abc}	7.437^{a}
6	WBY	7.000^{cde}	7.125^{cd}	6.625^{bc}	6.875^{ab}	7.062^{a}	7.125^{bc}	7.187^{a}
7	WABK	7.437^{abcd}	7.375^{abcd}	6.875^{b}	7.000^{a}	7.000^{a}	7.500^{abc}	7.250^{a}
8	WABY	7.062^{cde}	7.062^{cd}	6.937^{ab}	6.875^{ab}	6.875^{a}	7.375^{abc}	7.250^{a}
9	BWBK	6.937^{de}	7.250^{abcd}	7.000^{ab}	6.875^{ab}	7.125^{a}	7.062^{c}	7.125^{a}
10	BWBY	6.562^{e}	6.312^{e}	5.875^{d}	5.937^{c}	5.625^{b}	5.500^{e}	5.937^{b}
11	BWABK	6.937^{de}	6.875^{de}	6.812^{bc}	7.125^{a}	6.875^{a}	7.562^{abc}	7.250^{a}
12	BWABY	7.312^{abcd}	7.375^{abcd}	7.312^{ab}	7.312^{a}	7.312^{a}	7.812^{a}	7.500^{a}
13	MzBK	7.750^{a}	7.562^{abc}	6.937^{ab}	7.000^{a}	6.937^{a}	7.437^{abc}	7.187^{a}
14	MzBY	7.687^{ab}	7.750^{ab}	7.062^{ab}	7.062^{a}	7.312^{a}	7.500^{abc}	7.375^{a}
15	MzABK	7.875^{a}	7.812^{a}	7.062^{ab}	7.125^{a}	7.125^{a}	7.500^{abc}	7.250^{a}
16	MzABYY	7.375^{s}	7.500^{abc}	7.312^{ab}	7.062^{a}	6.937^{a}	7.125^{bc}	7.180^{a}

Mean with the same superscript letter within each column are not significantly different from each other at the 5% level ($P < 0.5$). N=256: 1-9 point hedonic scoring scale."

4.6 Shelf life study of cookies:

Various tests were conducted over a period of 60 days to predict the shelf life of cookies, including microbial counting on PCA and PDA, moisture content, and sensory evaluation. With the exception of the BWBY sample, the initial moisture content of the cookies was approximately 5.04%. This low moisture content was due to the high baking temperature of 200°C, which caused the sucrose to crystallize and release water vapor. However, cookies made with buckwheat had a higher moisture content because they absorbed more water during dough mixing, resulting in lower sensory acceptance compared to the other cookie samples.

4.7 Carbohydrate tests of cookies:

Many carbohydrate detection tests, including Molisch's, Iodine's, Seliwanoff's, Fehling's, Barfod's, and Bial's tests, were conducted on the cookie samples. The results revealed that all of the samples had positive outcomes, indicating the presence of various types of carbohydrates such as starch, reducing monosaccharides, pentose sugars, and ketose sugars in the cookies.

Table 13: Qualitative test for the carbohydrates in cookies

Sample	Molisch's test	Iodine's test	Seliwanoff's test	Fehling's test	Barfod's test	Bial's test
MBK	+	+	+	+	+	+
MBY	+	+	+	+	+	+
MABK	+	+	+	+	+	+
MABY	+	+	+	+	+	+
WBK	+	+	+	+	+	+
WBY	+	+	+	+	+	+
WABK	+	+	+	+	+	+
WABY	+	+	+	+	+	+
BWBK	+	+	+	+	+	+
BWBY	+	+	+	+	+	+
BWABK	+	+	+	+	+	+
BWABY	+	+	+	+	+	+
MzBK	+	+	+	+	+	+
MzBY	+	+	+	+	+	+
MzABK	+	+	+	+	+	+
MzABY	+	+	+	+	+	+
Control	+	+	+	+	+	+

CHAPTER V

DISCUSSION

In this study, locally available, various nutritious gluten-free hill grain flours such as millet, maize, buckwheat, and taichin rice flour were used instead of refined wheat flour (Maida flour) as the main ingredient for the production of gluten-free cookies. Whole grain wheat flour was also used as a control for the development of low-gluten cookies. In addition, a ready-made cookie made from all-purpose flour (Maida) was also purchased from the market as a control sample.For the development of gluten free cookies, refined wheat flour was fully replaced with hill grain flours and glutinous rice flour (taichin rice flour) in 3:1 ratio that is 375 gm millet flour with 125 gm taichin rice flour. Glutinous rice flour, which has a higher concentration of the starch amylopectin, was added to improve the viscoelasticity of the cookies. Although starch is not soluble in water, it absorbs water and swells, which increases the volume of the cookies. According to a study by Qin et al (2016) demonstrating that, the dry-heat treatment had a greater impact on the glutinuous rice flour (GRF) and a higher viscoelasticity in food industry.

In modern diets, cookie consumption is increasing. One of the main ingredients used to make traditional cookies is refined wheat flour (maida). This flour undergoes chemical bleaching, which eliminates all of the dietary fiber, minerals, and vitamins, leaving behind a product that is heavy in carbohydrates. Traditional cookies produced with this type of flour are deficient in fiber and vital amino acids, but they are high in fat and carbohydrates which increased the risk of diabetes acccording to earlier studies by Goswami et al (2021). Thus the nutritional content of these cookies such as ash content of developed cookies were increased (from 0.8% to 1.75%) and fiber content (upto 7.8%) were increased by substituting the wheat flour with millet flour ,maize flour, buckwheat flour,glutinous rice flour and whole grain wheat flour instead of refine wheat flour (Rathi et al 2004 and Kulthe et al 2018).

Another significant advantage of substituting the refined wheat flour by cereals flour in cookie production is the presence of gluten protein. In order to address this issue, a study was conducted to develop a gluten-free cookie recipe that eliminates this protein. Gluten-related disorders have become a prevalent concern in recent times, as gluten can cause celiac disease, gluten allergies, and non-celiac gluten sensitivity, which can damage the lining of the small intestine. A gluten-free diet is often the most effective treatment for these disorders. However, creating a gluten-free bakery product can be a difficult task for the food industry, as wheat protein has unique viscoelastic properties and the ability to form cohesive dough (Gallagher et al 2004; Day et al 2006; Altındağ et al 2014).

A total of 16 different cookie samples were created in this research by altering the leavening agent, adding fungal alpha-amylase enzyme, and replacing traditional flour with various cereal flours. In conventional cookie production, only baking powder is utilized as a leavening agent, but in this study, in addition to baking powder, baker's yeast and fungal alpha-amylase enzyme were also employed to develop a unique and new product. In this particular recipe,both baking soda (Sodium bicarbonate) and baking powder were used. When baking soda alone was insufficient to produce enough carbon dioxide for leavening, this combination often produced additional lift throughout the baking process. when gluten was removed from flour, it reduced the flour's ability to create a structure that could hold gases during the fermentation process of bread dough. To improve this, enzymes were added to the gluten-free dough to enhance its viscoelastic properties (Eduardo et al 2013; Schoenlechner et al 2013). Thus, gluten-free dough required additional fungal alpha amylase treatment in order to improve its physical properties to be comparable to those of gluten-containing dough.

According to the presented formulation recipe (table 3,4,5) it could be observed around 2 % baker’s yeast and 0.02% food-grade improver with fungal alpha-amylase enzyme was used as leavening agent and dough improver instead of baking powder. Typically, the yeast flavor is not detectable in the baked items if the amount of yeast added is less than 2.5% of the baker's total ingredients (Linda and Stanley 2007). By incorporating the amylase enzyme and instant baker's yeast in gluten free composite flours and keeping it warm and humid in a proofer for 1 hour, the flours and starches were able to absorb more liquid. The solution to the gritty texture issue in gluten-free cookies was achieved by altering the recipe for traditional cookies, which also improved the quality of the dough through the amylolytic process and fermentation process. Javaria et al (2016) discovered that utilizing sourdough fermentation or biological leavening with baker's yeast significantly impacted the texture and quality of gluten-free baked goods. The researchers observed that combining gluten-free flour, water, and other ingredients, followed by fermenting with lactic acid bacteria (LAB) derived from an overnight culture to attain a concentration of roughly 5.0E+07 cells per gram or supplementing with baker's yeast (1.25%), considerably improved the quality of the bread dough.

The study found that among the 16 cookie samples tested, the Buckwheat-taichin rice flour formulated gluten-free cookie sample had the highest ash content at 2.59%. The second and third highest ash content was found in BWABK and WABK at 2.57% and 2.36% respectively. The lowest ash content was found in the control (C1) and MzABK coded sample at 0.81% and 1.36%. Comparison of the two control cookies revealed that the cookie made with whole grain wheat flour had a higher ash content than the cookie made with refined wheat flour. The study concluded that the ash content in cookies had risen from 0.81% to 2.59% due to the substitution of refined wheat flour with high amount ash content wholegrain wheat flour, millet flour, maize flour, buckwheat flour and glutinous rice flour. Among the millet, maize and buckwheat based cookies, buckwheat based cookies had the highest ash content, followed by millet based cookies and then maize based cookies. Similar observation was stated by Bhavsar et al (2013). The elevated ash content observed in these cookies indicates that they contain higher amounts of minerals than conventional cookies. According to research by Suriya et al. (2017), cookies composed solely of refined wheat flour (C1) had an approximately equal amount of ash as refined wheat flour, which had an ash level of 0.75%. The higher ash content in these cookies suggested that they contain more minerals than traditional cookie. The ash content in cookies made from of millet-taichin rice flour (75%:25%) and maize-taichin rice flour (75%:25%) was found to be twice as high as that of traditional cookies made with only refined wheat flour (C1). However, cookies made from a mixture of buckwheat-taichin rice flour(75%:25%) was found to have almost four times than the ash content of traditional cookies. This is in agreement with previous studies by Bhavsar et al (2013) and Rai et al (2014) which reported similar ash content in buckwheat flour, maize flour, millet flour, and rice flour.

The moisture content of gluten-free cookies was measured at various intervals (0, 30, and 60 days) and the results showed a range of 1.05% to 8.17% across different samples. With the exception of MABY, which remained constant, the moisture content of all samples tended to rise over time. The control sample had a moisture content of 2.10% at 30 days. The sample of BWBY had the most significant increase in moisture content over the 60 days, going from 6% to 8.67%, this is an increase of 44.5%. Some samples, like MBK, saw a small rise in moisture content, from 1.15% to 2% at 60 days of storage. However, some, like MABY, maintained a constant moisture content of 2% throughout the intervals while others, such as BWBY, showed a significant increase with time. The study showed that the moisture content of most samples generally increases over time. Due to the hygroscopic nature of cookies, they tend to absorb moisture from the air during storage, and the moisture content significantly increased over time. This has an impact on the quality and safety of cookies. The highest moisture content in the 16 formulated cookie samples was found to be below 10% until 60 days of storage at room temperature (25 ± 2°C), which reduced the chance of microbial deterioration. These observations were in agreement with the earlier reports (Ayo et al 2007) in that low moisture content of less than 10% minimized the risk of microbial spoilage and extended the shelf life of cookies. Another study by (Adeleke and Odedeji 2010; Ogunlakin et al 2012) suggested that a moisture content below 14% is ideal for long-term storage. Adebowale et al (2012) also found that baked goods like cakes, cookies, and bread with high moisture content are prone to bacterial, yeast, and mold growth, leading to spoilage.

Microbial analysis was conducted on formulated cookies that were stored in a recyclable PET plastic tray and packaged in a polypropylene plastic bag at room temperature (25 ± 2°C) for 60 days. The examination consisted of a total viable count on Plate Count Agar and a yeast and mold count on Potato Dextrose Agar. At the start of the study, no bacterial or fungal colonies were found on either the PCA or PDA plates . After 30 days of storage, a detectable number of organisms were found on the PCA plate and some fungal colonies were observed in certain samples (MBK, MBY, MABK, MABY), while no growth of yeast or mold was observed in other samples (WBK, WBY, WABK, WABY, BWBK, BWBY, BWABK, BWABY, MzBK, MzBY, MzABK, MzABY). However, by 60 days, all the samples showed Yeast and Mould Count (YMC) values ranging from 2.0 x 10^2 cfu/gm to 1.3 x 10^3 cfu/gm. The control sample had a YMC of 1.2 x 10^3 cfu/gm on day 30 of storage and a similar amount of YMC was observed in 3 test samples (MBY, MABY, and MzABK) on day 60. As per the 1994 standards set by the World Health Organization (WHO), the acceptable limit for total plate count (TPC) in baked goods should not exceed 2.0x10^5 cfu/gm, and the the maximum acceptable limit for yeast and mold should be less than 1.0x10^4 cfu/gm. This result revealed that the overall Yeast and Mould count (YMC) was within the acceptable limit, and the developed cookies were safe for consumption. The study found that the yeast and mold content (YMC) in gluten free cookie increased slightly after 60 days as compared to 30 days. The results suggested that the type of gluten-free flour used, storage conditions, type of packaging materials, and other elements that could affect yeast and mold growth in gluten-free cookies ,which also impact the shelf life and safety of the product.

At the beginning of the storage period (0 days), no visible bacterial or fungal growth was observed on both PCA and PDA plates, indicating that the cookies, which underwent a dry heat treatment during baking, were free from microbial contamination. However, after 30 and 60 days of storage, there was a significant increase in the populations of viable bacteria and fungi. This increase could be attributed to post contamination from the packaging materials used during the packaging . Comparing the storage periods of 0 days and 60 days, it is evident that there is directly association between storage duration and the growth of microorganisms. The quality of the cookies during storage is influenced by the packaging materials employed. This finding was supported by Anggraini and Salam (2021), which suggested that that polyethylene (PE) plastic has lower permeability than polypropylene (PP), allowing a higher amount of water vapor to enter the packaging material over storage time. The mean bacterial count of the cookies sample on PCA after 30 days of storage was found to range from 1.4 x 10^2 to 2.37 x 10^3 cfu/gm and after 60 days it increased to 1.8 x 10^2 to 4.16 x 10^3 cfu/gm. According to a study by Banusha and Vasantharuba (2014), the bacterial count in cookies should not exceed $1x10^4$ CFU/gm to be considered safe for consumption. The results of the microbiological examination indicated that the overall bacterial count in the cookie samples was below this threshold and therefore, the cookies were safe to eat. The increase in the count of viable microorganisms in most of the test samples after 60 days of storage compared to 30 days could be attributed to factors such as the lack of preservatives, hygroscopic nature of cookies, low-grade packaging and storage conditions (temperature of 25 ± 2°C) that are favorable for the growth of microorganisms. The control sample had relatively lower viable microorganism count compared to most of the test samples after 60 days of storage, which could be due to the addition of the antioxidant Tert-butyl hydroquinone (E319) to protect against oxidative processes in the cookie and prevent the proliferation of various microbes during production of control cookie. Similar findings were published by Khezerlou et al (2022), where Tert-butyl hydroquinone (TBHQ) is utilized to prevent spoiling or rancidity in foods such as lipids, edible oils, cereals, and shortening as major ingredient due to its antioxidant and antibacterial capabilities, with low impact on food sensory aspects.

The sensory attributes of the cookies were rated using a sensory evaluation score of 1-9 hedonic scale, with 9 being the highest score. Based on the data, the overall acceptability score of the cookies were decreased over time.. Acoording to table 12,13,14, cookies prepared from millet-taichin rice flour , buckwheat-taichin rice flour and Maize-taichin rice flour were achieved almost similar score (above 7) for all the organoleptic parameters except BWBY sample. The cookies with the highest OAC score at 0 day were WBK (8.250) and WBY (8.125). The cookies with the lowest OAC score at 0 day was BWBY (6.625).The cookies with the highest OAC score at 60 days were

WBK (7.437) and MABK (7.437). The cookies with the lowest OAC score at 60 days were BWBY (5.937). In a study conducted by Chopra et al (2014), a comparable tendency was observed. When the concentration of buckwheat flour was increased to 100%, it resulted in a decrease in the scores for top grain, appearance, texture, and flavor, with a score of 5.25. This decline was caused by the formation of cracks in the gluten-free buckwheat flour. Furthermore, the flavor score dropped significantly to 5.71 at higher concentrations, which could be attributed to the presence of bitter-tasting flavonoid compounds like rutin in buckwheat flour. However, cookies made with a combination of 75% buckwheat flour and 100% wheat flour received high scores for overall acceptability.

The OAC score of the gluten-free millet- taichin flour cookies sample coded MBK (7.437) was comparable to the control sample after 30 days of storage. An analogous result was observed in research done by Swami et al (2013). The findings of the sensory evaluation showed that the cookies made with finger millet flour, which had a 30% oil content, and cooked at 200°C for 20 minutes received higher ratings (7 out of 9) for their texture, color, and overall acceptability. However, after 60 days of storage, the sensory attributes such as taste, texture, flavor, crunchiness, and overall acceptability was dropped marginally. This decline could be due to the hygroscopic nature of cookies, low-quality packaging, absence of preservatives, and improper storage conditions. Additionally, packaging the cookies in metalized polyester polyethylene (MET-PPE) pouches instead of polypropylene (PP) plastic pouches would have improved their durability and maintained their crispiness. Jan et al (2017) supported this finding by demonstrating that low-density polyethylene (LDPE) pouches exhibited greater moisture absorption, higher levels of free fatty acids, peroxide value, and microbial growth compared to LP and MET-PPE pouches. Therefore, MET-PPE packaging can be utilized for storing cookies under ambient conditions (25 ± 2°C) for up to 4 months without compromising their crunchiness or other quality attributes.

This study found that cookies treated with food grade fungal α-amylase with improver and either baker's yeast or baking powder were more preferred by panelists in terms of crunchiness, taste, texture, color, and flavor than cookies treated with only baking powder or baker's yeast. The combination of the food grade fungal amylase enzyme with baker's yeast or baking powder helps to mitigate the issue of a gritty texture in gluten-free products and improves the color, flavor, and taste of the cookies. Javaria et al (2016) suggested that the use of biological leavening with baker's yeast or sourdough fermentation has a significant impact on the texture and quality of gluten-free baked goods. The sensory characteristics of cookies were greatly influenced by factors such as the time and temperature for proofing, the amount of water added during dough mixing, the baking time and temperature, the type of flours used, and the packaging materials. Swami et al (2013) found that the cookies prepared with a 30% oil content and baked at 200°C for 20 minutes showed a greater level of acceptance in overall cookie quality compared to cookies made with different oil content and baking temperatures.

All types of carbohydrates, including starch, reducing monosaccharides, pentose sugars, ketose sugars, oligosaccharides, polysaccharides, reducing sugar, and non-reducing sugar, were present in all of the formulated cookies. In comparison between test samples and contro sample, there was found no significant difference in all of carbohydrates tests.

Creating gluten-free cookies that have positive sensory attributes is a significant challenge. Gluten-free products often lack the appearance, color, texture, smell, and flavor of products made without wheat flour, leading to lower sensory acceptance. However, this novel formulation recipes that combines hill grain flours (such as maize, millet, and buckwheat) and taichin rice flour (3:1) has been found to be a major breakthrough in the gluten-free production industry. The cookies made from this recipes were confirmed to be microbiologically safe as well as highly scored in terms of sensory appeal. The study showed that cookies made from a combination of millet-taichin rice flour, maize-millet rice flour, buckwheat-rice flour, and whole grain wheat-rice flour (as a control) were generally accepted. Therefore, this formulation recipe has the great potential to develop nutritious gluten-free cookies that can assist consumers to manage the gluten-related diseases.

CHAPTER VI

CONCLUSION AND RECOMMENDATIONS

6.1 Conclusion

Novel recipes for gluten free cookies were successfully developed by replacing refined wheat flour with the hill grains (maize,millet,buckwheat and taichin rice flour) of Nepal. The research determined that adding such a low-cost, highly nutritious, and locally available composite flours to gluten-free cookie recipes instead of refined flour would not only increase their nutritional value but also enhance their texture and sensory appeal. The study concluded that the inclusion of food grade fungal alpha-amylase along with baking powder or baker's yeast in the production of cookies broke down the starch-rich flour into dextrin, created enzymatic browning, enhanced flavor development, resolved the gritty texture issue and improved water distribution in the dough. This research also come to conclusion that the shelf life of formulated cookies can be prolonged by using metalized polyester polyethylene (MET-PPE) pouches or polyethylene (PE) plastic bag for packaging instead of polypropylene (PP) pouches. Additionally, incorporating preservatives can further extend the shelf life of the cookies. Thus, these cookies can be used as dietary snack for gluten related disorder suffers.

6.2 Recommendations

1. Developing gluten-free cookies by completely replacing traditional refined flour with gluten-free grain flour is in itself a challenge. However, this novel formulation of gluten-free cookie recipes could be a landmark in the bakery industry of Nepal.
2. There are many locally available, underutilized, and inexpensive nutritious hill grains in Nepal, such as millet, maize, buckwheat, and taichin rice, that can be utilized in the creation of gluten-free cookies.
3. Fungal α-amylase might be useful as dough improver to solve the problem of gritty texture in gluten free cookies.
4. Preservative such as antioxidant and high grade packaging such as metalized polyester polyethylene (MET-PPE) pouches instead of polypropylene (PP) plastic pouches could be helpful to maintain the crunchiness of cookies and extend its shelf life.
5. It is possible to substitute chemical leavening agents like baking powder with biological leavening agents such as baker's yeast, to improve the taste, texture, and color of gluten-free cookies.
6. Further investigation is needed for substituting sugar with alternative natural sweeteners, to develop sugar-free gluten-free cookies for diabetic suffers.

REFERENCES

Abiramy NS, Dalukdeniya DACK and Abirajitha M (2017). Development and quality evaluation of rice (*Oryza sativa*) flakes incorporated gluten free cookies from maize (*Zea mays* l.) and soy bean (*Glycine max*) flour blends. 03 Annual Research Session rd.

Adebowale AA, Adegoke MT, Sanni SA, Adegunwa MO and Fetuga GO (2012). Functional properties and biscuit making potentials of sorghum-wheat flour composite. American Journal of food technology **7**(6): 372-379.

Adeleke RO and Odedeji JO (2010). Functional properties of wheat and sweet potato flour blends. Pakistan Journal of Nutrition **9**(6): 535-538.

Akbar K and Ayub M (2018). Effect of Storage on the Quality of Wheat and Maize Based Cookies. Sarhad Journal of Agriculture **34**(3).

Altındağ G, Certel M, Erem F and İLknur Konak L (2014). Quality characteristics of gluten-free cookies made of buckwheat, corn, and rice flour with/without transglutaminase. Food Science and Technology International **21**(3): 213–220.

Alvarez-Jubete L, Arendt E and Gallagher E (2010). Nutritive value of pseudocereals and their increasing use as functional gluten-free ingredients. Trends in Food Science & Technology **21**(2): 106–113.

Ananingsih VK, Gao J and Zhou W (2012). Impact of Green Tea Extract and Fungal Alpha-Amylase on Dough Proofing and Steaming. Food and Bioprocess Technology **6**(12): 3400–3411.

Aneja KR (2007). Experiments in microbiology, plant pathology and biotechnology. New Age International, pp 278-282.

Anggraini PDK and Salam A (2021). Acceptance, nutritional content, and shelf life of cookies based on millet flour as a functional food. Technium Soc. Sci. J 21:795.

AOAC official methods of analysis (1980). 15th edition, Association of official analytical chemists, Washington, DC.

Awolu OO, Omoba O, Olawoye O and Dairo M (2016). Optimization of production and quality evaluation of maize-based snack supplemented with soybean and tiger-nut (Cyperus esculenta) flour. Food Science and Nutrition **5**(1): 3–13.

Ayo JA, Ayo VA, Nkama I and Adewori R (2007). Physiochemical, in-vitro digestibility and organoleptic evaluation of. Nigerian food journal **25**(1):77-89.

Baljeet SY, Ritika BY, Roshan LY (2010). Studies on functional properties and incorporation of buckwheat flour for biscuit making. J Int. Food Res. 17: 1067-1076.

Balyan HS, Gupta PK, Kumar S, Dhariwal R, Jaiswal V, Tyagi S, Agarwal P, Gahlaut V and Kumari S (2013). Genetic improvement of grain protein content and other health-related constituents of wheat grain. Plant Breeding.

Banusha S and Vasantharuba S (2014). Preparation of wheat-malted flour blend biscuit and evaluation of its quality characteristics. American Eurasian Journal of Agriculture and Environmental Sciences 14: 459-463.

Bathla S, Jaidka M and Kaur R (2019). Nutritive Value. InmMaize Production and Use. IntechOpen.

Bhavsar GJ, Sawate AR, Kshirsagar RB and Chappalwar VM (2013). Studies on Physico-Chemical Characteristics of Buckwheat and Its Exploration in Bread as Functional Food. International Journal of Engineering Research and Technology **2**(11).

Brites LTGF, Ortolan F, Silva DWD, Bueno FR, Rocha TDS, Chang YK, and Stela CJ (2019). Gluten-free cookies elaborated with buckwheat flour, millet flour and chia seeds. Food Science and Technology **39**(2): 458–466.

Brodie J (2006). Chemical Leavening for Baking. Formulating Bakery products, International seminar, AIB International, Manhattan, KS, USA

Catassi C and Fasano A (2008). Celiac disease. In Gluten-Free Cereal Products and Beverages. Arendt EK and Del BF (eds). Academic Press, USA, pp 1–27.

Catassi C, Bai J, Bonaz B, Bouma G, Calabrò A, Carroccio A, Castillejo G, Ciacci C, Cristofori F, Dolinsek J, Francavilla R, Elli L, Green P, Holtmeier W, Koehler P, Koletzko S, Meinhold C, Sanders D, Schumann M and Fasano A (2013). Non-Celiac Gluten Sensitivity. The New Frontier of Gluten Related Disorders. Nutrients **5**(10): 3839–3853.

Chakraborty R, Sabruna S, Roy R, Majumdar S and Roy S (2021). Banana pseudostem substitution in wheat flour biscuits enriches the nutritional and antioxidative properties with considerable acceptability. SN Applied Sciences **3**(1).

Cheng YF and Bhat R (2016). Functional, physicochemical and sensory properties of novel cookies produced by utilizing underutilized jering (*Pithecellobium jiringa* (Jack)) legume flour. Food Bioscience 14: 54–61.

Chiotellis E and Campbell G (2003). Proving of Bread Dough I. Food and Bioproducts Processing, **81**(3), 194–206.

Chopra N, Dhillon B and Puri S (2014). Formulation of buckwheat cookies and their nutritional, physical, sensory, and microbiological analysis. The International Journal of Advanced Biotechnology Research (IJABR) **5**(3): 381-387.

Chopra N, Dhillon B, Rani R and Singh A (2018). Physico-Nutritional and Sensory Properties of Cookies Formulated with Quinoa, Sweet Potato and Wheat Flour Blends. Current Research in Nutrition and Food Science **6**(3): 798-806 .

Day L, Augustin M, Batey I and Wrigley C (2006). Wheat-gluten uses and industry needs. Trends in Food Science & Technology **17**(2): 82–90.

De Leyn I (2014). Other leavening agents. In Bakery products : science and technology. Zhou W , Hui Y, De Leyn I, Pagani M, Rosell C, Selman J and Therdthai N. (eds) pp 175–181.

Delcour JA, Joye IJ, Pareyt B, Wilderjans E, Brijs K and Lagrain B (2012). Wheat gluten functionality as a quality determinant in cereal-based food products. Annual review of food science and technology 3: 469-492.

Dhillon B, Wiesenborn D, Sidhu H and Wolf- Hall C (2012). Improved microbial quality of Buckwheat using antimicrobial solution in a fludized bed. J Food Sci. 77: 98- 103.

Eduardo M, Svanberg U, Oliveira J and Ahrné L (2013). Effect of Cassava Flour Characteristics on Properties of Cassava-Wheat-Maize Composite Bread Types. International Journal of Food Science 2013:1–10.

Elli L, Branchi F, Tomba C, Villalta D, Norsa L, Ferretti F, Roncoroni L and Bardella MT (2015). Diagnosis of gluten related disorders: Celiac disease, wheat allergy and non-celiac gluten sensitivity. World Journal of Gastroenterology **21**(23): 7110–7119.

Fabjan N, Rode J, Košir IJ, Wang Z, Zhang Z and Kreft I (2003). Tartary Bucrakwheat *(Fagopyrum tataricum* Gaertn.) as a Source of Dietary Rutin and Quercitrin. Journal of Agricultural and Food Chemistry **51**(22): 6452–6455.

Ficco DB, De Simone V, De Leonardis AM, Giovanniello V, Del Nobile MA, Padalino L, Lecce L, Borrelli GM and De Vita P (2016). Use of purple durum wheat to produce naturally functional fresh and dry pasta. Food chemistry 205: 187–195.

Gallagher E, Gormley T and Arendt E (2004). Recent advances in the formulation of gluten-free cereal-based products. Trends in Food Science and Technology **15**(3):143–152.

Galić K, Ćurić D and Gabrić D (2009). Shelf Life of Packaged Bakery Good s—A Review, Critical Reviews in Food Science and Nutrition 49:5, 405-426.

Gally T, Rouaud O, Jury V, Havet M, Ogé A and Le-Bail A (2017). Proofing of bread dough assisted by ohmic heating. Innovative Food Science and Emerging Technologies 39: 55–62.

Gandhi AP, Kotwaliwale N, Kawalkar J, Srivastav DC, Parihar VS and Raghunadh P (2001). Effect of incorporation of defatted soy flour on the quality of sweet biscuits. J. Food Sci.Techno **38**(5): 502-503.

Goswami M, Sharma BD, Mendiratta SK and Pathak V (2021). Quality improvement of refined wheat flour cookies with incorporation of functional ingredients. Journal of Food Processing and Preservation **45**(4).

Granato D and Ellendersen LSN (2009). Almond and peanut flours supplemented with iron as potential ingredients to develop gluten-free cookies. Food Science and Technology **29**(2): 395-400.

Gujral HS, Haros M and Rosell CM (2003). Starch Hydrolyzing Enzymes for Retarding the Staling of Rice Bread. Cereal Chemistry Journal **80**(6): 750–754.

Ho LH, Abdul Aziz NA and Azahari (2013). Physico-chemical characteristics and sensory evaluation of wheat bread partially substituted with banana (Musa acuminata X balbisiana cv. Awak) pseudo-stem flour. Food Chemistry **139**(1): 532–539.

Hossain A, Basak P, Azam G, Kamal M, Rahman MM, Hasan MH and Islam R (2021). Study on Preparation and Development of Cookies Enriched with Mint Leaf (Mentha) Powder. EAS Journal of Nutrition and Food Sciences.

Hurs H and Martins S (2005). Low carbohydrate and beyond: The health benefits of Insulin. Cereal Food World 50: 57-60.

Hussain S, Mohamed AA, Alamri MS, Ibraheem MA, Abdo Qasem AA, Serag El-Din MF and Almaiman SAM (2020). Wheat–millet flour cookies:Physical, textural, sensory attributes and antioxidant potential. Food Science and Technology International **26**(4): 311-320.

Jan R, Saxena DC and Singh S (2017). Effect of storage conditions and packaging materials on the quality attributes of gluten-free extrudates and cookies made from germinated *chenopodium* (*Chenopodium album*) flour. Food Measure 11: 1071–1080.

Javaria S, Marwat SK, Raza S, Hameed A and Waseem K (2016). Formulation of gluten-free baked products for coeliac patients: a review of contemporary methodologies and quality improving factors. American Eurasian J Agric and Environ Sci **16**(4): 826-835.

Jiamjariyatam R, Krajangsang S and Lorliam W(2022). Effects of Jasmine Rice Flour, Glutinous Rice Flour, and Potato Flour on Gluten-Free Coffee Biscuit Quality. Journal of Culinary Science & Technology 1-19.

Karn SK (2017). Food Composition Table (Third Edition). Department of Food Technology and Quality Control,Babarmahal, Kathamandu pp 6-8.

Kataria R (2014). Proximate Nutritional Evaluation of Maize and Rice Gluten Free Cereal. IOSR Journal of Nursing and Health Science **3**(2) : 01–06.

Kato N, Kayashita J and Tomotake H (2001). Nutritional and physiological functions of buckwheat protein. Recent Research Development Nutrition 4: 113–119.

Khairuddin MAN and Lasekan O (2021). Gluten-Free Cereal Products and Beverages: A Review of Their Health Benefits in the Last Five Years. Foods **10**(11): 2523.

Khezerlou A, Akhlaghi AP, Alizadeh AM, Dehghan P and Maleki P (2022). Alarming impact of the excessive use of tert-butylhydroquinone in food products: A narrative review. Toxicology Reports 9:1066–1075.

Khoury DE, Balfour DS and Joye IJ (2018). A Review on the Gluten-Free Diet: Technological and Nutritional Challenges. Nutrients **10**(10): 1410.

Kulkarni DB, Sakhale BK and Chavan RF (2021). Studies on development of low gluten cookies from pearl millet and wheat flour. Food Research **5**(4): 114-119.

Kulthe AA, Thorat SS and Khapre AP (2018). Nutritional and sensory characteristics of cookies prepared from pearl millet flour. The Pharma Innovation Journal **7**(4): 908-913.

Linda Y and Stanley PC (2007). Technology of Breadmaking. Springer, Berlin. pp 79.

Luang-In V, Yotchaisarn M, Saengha W, Udomwong P, Deeseenthum S and Maneewan K (2019) . Isolation and Identification of Amylase-producing Bacteria from Soil in Nasinuan Community Forest, Maha Sarakham, Thailand. Biomed Pharmacol J **12**(3).

Luitel DR, Siwakoti M and Jha PK (2020). Nutrients in finger millet and soil at different elevation gradients in Central Nepal. CABI Agriculture and Bioscience **1**(1).

Luitel DR, Siwakoti M, Jha PK, Jha AK and Krakauer N (2017). An Overview: Distribution, Production, and Diversity of Local Landraces of Buckwheat in Nepal. Advances in Agriculture pp 1–6.

Manley D. (2000). Technology of biscuits, crackers and cookies. 3rd edition. Cambridge, UK,Woodhead.

Martínez E, Oliveira MBP, Rabadán A, Millan CA and Pardo JM (2022). Elaboration of Cookies Using Oils and Flours from Seeds and Nuts: Effects on Technological, Nutritional and Consumer Aspects. Foods **11**(15): 2249.

Michaelraj PS and Shanmugam A (2013). A Study on Millets Based Cultivation and Consumption in India. International Journal of Marketing, Financial Services and Management Research 2: 49-58.

Mobini-Dehkordi M and Javan FA (2012). Application of alpha-amylase in biotechnology. J. Biol. Today World **1**(1): 39-50.

Molinari R, Costantini L, Timperio AM, Lelli V, Bonafaccia F, Bonafaccia G and Merendino N (2017). Tartary buckwheat malt as ingredient of gluten-free cookies. Journal of Cereal Science 80: 37–43.

Muyanja C, Gonahasa J, Nabakooza J and Byamukama J (2020). Maize germ and bran for value addition: high fiber bakery and confectionery products. Scinnovent Center Policy Brief, No. 8/2020.

Neeharika B (2020). Leavening Agents for Food Industry. International Journal of Current Microbiology and Applied Sciences **9**(9): 1812–1817.

Ogunlakin GO, Oke MO, Babarinde GO and Olatunbosu DG (2012). Effect of drying methods on proximate composition and physico-chemical properties of cocoyam flour. American Journal of Food Technology 7: 245-250.

Oort MV (2010). Enzymes in bread making. In Enzymes in food technology. Whitehurst RJ and Oort MV (eds).Chichester,Wiley, pp 103–140.

Otero-Guzmán NC, Rodríguez-Sandoval E and Tabares-Londoño JA (2020). Influence of different types of baking powder on quality properties of muffins. Dyna **87**(214): 9-16.

Peter Ikechukwu A, Okafor DC, Kabuo NO, Ibeabuchi JC, Odimegwu EN, Alagbaoso SO and Mbah RN (2017). Production and evaluation of cookies from whole wheat and date palm fruit pulp as sugar substitute. International Journal of Advancement in Engineering Technology, Management and Applied Science **4**(04): 1-31.

Pop G (2007). Researches regarding the chemical leavening agent's role in quality of bakery products. Journal of Agroalimentary Processes and Technologies **13**(1): 105-112.

Poutanen K, Flander L and Katina K (2009). Sourdough and cereal fermentation in a nutritional perspective. Food microbiology **26**(7): 693-699.

Qin Y, Liu C, Jiang S, Cao J, Xiong L and Sun Q (2016). Functional Properties of Glutinous Rice Flour by Dry-Heat Treatment. PLoS One.

Qiu S, Abbaspourrad A and Padilla-Zakour OI (2021). Changes in the Glutinous Rice Grain and Physicochemical Properties of Its Starch upon Moderate Treatment with Pulsed Electric Field. Foods **10**(2): 395.Qin Y, Liu C, Jiang S, Cao J, Xiong L and Sun Q (2016). Functional Properties of Glutinous Rice Flour by Dry-Heat Treatment. PLoS One. **11**(8): 18.

Rai S, Kaur A and Singh B (2014). Quality characteristics of gluten free cookies prepared from different flour combinations. Journal of Food Science and Technology **51**(4): 785–789.

Ranganna S (2012). Handbook of Analysis and Quality Control of Fruits and Vegetable Products.Tata McGerw Hill Publication Co,New Delhi.

Rai BK and K.C JB (2007). Moisture in foods. In Essentials of Food Chemistry.1st edition,Maya K.C publication,Kathmandu, pp 201-202.

Rao BD, Kulkarni DB, Kavitha C (2018). Study on evaluation of starch, dietary fiber and mineral composition of cookies developed from 12 sorghum cultivars. Food Chemistry 238: 82–86.

Rathi A, Kawatra A, Sehgal S and Housewright B (2004). Influence of depigmentation of pearl millet (Pennisetum gluacum L.) on sensory attributes, nutrient composition and in vitro digestibility of biscuits. Lebensm. Wiss. Technol 37: 187-192.

Rostami NM, Rostami K, Pourhoseingholi MA, Nazemalhosseini ME, Sanaei M, Dabiri H and Zali MR (2008). M1220 The Proportion of Celiac Disease in Common Gastroenteropaties Among Iranian Patients. Gastroenterology **134**(4): A-364.

Sakač M, Pestorić M, Mandić A, Mišan A, Nedeljković NN, Jambrec D, Jovanov P, Lazić V, Pezo L and Sedej I (2016). Shelf-life prediction of gluten-free rice-buckwheat cookies. Journal of Cereal Science 69: 336–343.

Sapone A, Bai JC, Ciacci C, Dolinsek J, Green PH, Hadjivassiliou M, Kaukinen K, Rostami K, Sanders DS, Schumann M, Ullrich R, Villalta D, Volta U, Catassi C and Fasano A (2012). Spectrum of gluten-related disorders: consensus on new nomenclature and classification.

Schiepatti A, Savioli J, Vernero M, Borrelli DAF, Perfetti L, Meriggi A and Biagi F (2020). Pitfalls in the Diagnosis of Coeliac Disease and Gluten-Related Disorders. Nutrients **12(**6): 1711.

Schoenlechner R, Szatmari M, Bagdi A and Tömösközi S (2013). Optimisation of bread quality produced from wheat and proso millet (*Panicum miliaceum* L.) by adding emulsifiers, transglutaminase and xylanase. Food Science and Technology **51**(1): 361–366.

Schopf M, Wehrli MC, Becker T, Jekle M and Scherf KA (2021). Fundamental characterization of wheat gluten. European Food Research and Technology 247: 985-997.

Schuppan D, Dennis MD and Kelly CP (2005). Celiac disease: Epidemiology, pathogenesis, diagnosis, and nutritional management. Nutrition in Clinical Care **8**(2): 54–69.

Sharma N, Bhatia S, Chunduri V, Kaur S, Sharma S, Kapoor P, Kumari A and Garg M (2020). Pathogenesis of Celiac Disease and Other Gluten Related Disorders in Wheat and Strategies for Mitigating Them. Frontiers in Nutrition 7 .

Shrestha B (2002). Pratical for Biochemistry and Biotechnology.SNEMP, pp 32-36.

Singh S, Riar CS and Saxena DC (2008). Effect of incorporating sweet potato flour to wheat flour on the quality characteristics of cookies. African journal of food science **2**(6): 65-72.

Sinha R and Sharma B (2017). Use of finger millet in cookies and their sensory and nutritional evaluation. Asian Journal of Dairy and Food Research **36**(03).

Stibilj V, Kreft I, Smrkolj P and Osvald J (2004). Enhanced selenium content in buckwheat (*Fagopyrum esculentum* Moench) and pumpkin (*Cucurbita pepo* L.) seeds by foliar fertilisation. European Food Research and Technology **219**(2).

Sundarram A and Murthy TPK (2014). α-amylase production and applications: a review. Journal of Applied & Environmental Microbiology, **2**(4): 166-175

Suriya M, Rajput R, Reddy CK, Haripriya S and Bashir M (2017). Functional and physicochemical characteristics of cookies prepared from *Amorphophallus paeoniifolius* flour. Journal of food science and technology **54**(7): 2156–2165.

Swami SB, Thakor N and Shinde UN (2013). Effect of Baking Temperature and Oil Content on Quality of Finger Millet Flour Cookies. Journal of Agricultural Engineering 50: 32–38.

Tanwar B and Dhillon M (2017). Preparation and nutritional quality evaluation of gluten-free cookies. Asian Journal of Dairy and Food Research **36**(01).

Taraghikhah N, Ashtari S, Asri N, Shahbazkhani B, Al-Dulaimi D, Rostami NM, Rezaei TM, Razzaghi MR and Zali MR (2020). An updated overview of spectrum of gluten-related disorders: clinical and diagnostic aspects. BMC Gastroenterology **20**(1).

Thompson T(2009). The nutritional quality of gluten free foods. Gluten-Free Food Science and Technology. 1st edition.Wiley-Blackwell. pp42-45.

Tiwari SP, Srivastava R, Singh CS, Shukla K, Singh RK, Singh P and Sharma R (2015). Amylases: an overview with special reference to alpha amylase. J Global Biosci **4**(1): 1886-1901.

Torbica A, Hadnađev M and Dapčević HT (2012). Rice and buckwheat flour characterisation and its relation to cookie quality. Food Research International **48**(1): 277–283.

U.S. Food and Drug Administration (2022). Questions and Answers on the Gluten-Free Food Labeling Final Rule.

https://www.fda.gov/food/food-labeling-nutrition/questions-and-answers-gluten-free-food-labeling-final-rule Accessed 25 April 2023

Wandhekar SS, Bondre N and Swami A (2021). Formulation and Quality Evaluation of Finger Millet (Ragi) Fortified Cookies. International Journal of Innovation, Leadership, Society and Sustainability **1**(1).

Wei Y, Hu X, Zhang G and Ouyang S (2003). Studies on the amino acid and mineral content of buckwheat protein fractions. Nahrung Food 47: 114-116.

Weiss GA (2021). Diagnosis and Management of Gluten-Associated Disorders: A Clinical Casebook .1st edition. Springer.

WHO (1994).Guideline value for food and drinking water. World Health Organization,Geneva. pp3-4.

WHO/FAO (2003). Diet, nutrition and the prevention of chronic diseases. Report of a WHO/FAO expect consultation, World Health Organization Technical report series 916, WHO ,Geneva.

Xu J, Zhang Y, Wang W and Li Y (2020). Advanced properties of gluten-free cookies, cakes, and crackers: A review. Trends in Food Science and Technology 103: 200–213.

Zannini E, Jones JM, Renzetti S and Arendt EK (2012). Functional Replacements for Gluten. Annual Review of Food Science and Technology **3**(1): 227–245.

Zlatanović S, Kalušević A, Micić D, Laličić PJ, Tomić N, Ostojić S and Gorjanović S (2019). Functionality and Storability of Cookies Fortified at the Industrial Scale with up to 75% of Apple Pomace Flour Produced by Dehydration. Foods **8**(11): 561.

Zouari R, Besbes S, Ellouze CS and Ghribi AD (2016). Cookies from composite wheat–sesame peels flours: Dough quality and effect of Bacillus subtilis SPB1 biosurfactant addition. Food Chemistry 194: 758–769.

APPENDIX-A

LIST OF EQUIPMENTS AND MATERIALS

A. Equipment's:

Aluminum Moisture Dishes

Autoclave

Bunsen burner

Egg shaker Model.no.OG-1004, 220-240V, 50/60 HZ, 550W

Grinding Machine

Incubator

Hot air oven

Microscope

Mortar and pestle

Muffle furnace

Porcelain crucible

Proofer Machine (automated)

Rotating Rack Convection Oven (digital)

Refrigerator

Spatula

Test tube stand

Tray (aluminum)

Weighing machine

Water bath

B. Glass wares and Plastic wares

Measuring cylinder	Test tubes
Beakers	Droppers
Petri-dishes	Pipettes
Conical Flasks	Volumetric flasks

C. Microbiological media

Plate Count Agar (PCA)
Potato Dextrose Agar (PDA)

D. Chemicals and Reagents

1% acetic acid solution
Molisch's Reagent
Conc.sulphuric acid (H2SO4)
Conc.hydrochoric acid (HCl)
0.33M copper (II) acetate
Resorcinol
Orcinol
10% ferric chloride
Iodine solution
Fehlings A solution
Fehlings B solution

E. Miscellaneous

Apron
Aluminum foil
Cotton wool
Distilled water
Gloves
Forceps
Permanent Marker
Lysol
Sanitizer

APPENDIX B

Scientific names and its nutritional benefits of different hill grains

S.N	Comman name /Nepalese name	Scientific name	Nutritional Benifits
1.	Buckwheat / Mithe Phaper	*Fagopyrum esculentum* Moench	Buckwheat has its unique nutrient composition that includes lysine rich protein, dietary fibre, mineral and trace elements, antioxidant rich vitamins and bioactive compounds such as rutin, quercetin and other flavonoids.
2.	Finger millet / kodo	*Eleusine coracana*	Finger millet is a highly nutritious grain and an excellent substitute to rice and wheat. It has high protein content (11%), low fat (4.2%) and very high fibre content (14.3%).They are rich in B vitamins, especially niacin, B6 and folic acid, as well as the minerals such as calcium, iron, potassium, magnesium and zinc. The phosphorus content in kodo millet is lower than any other millet and its antioxidant potential is much higher than any other millet and major cereals.
3.	Maize /Makai	*Zea mays*	Maize contains a number of important nutrition like; folic acid, pro-vitamin A (i.e. precursor to vitamin A), vitamin B1 (thiamine), vitamin B2 (niacin), vitamin B3 (riboflavin) vitamin B5 (pantothenic acid), vitamin B6 (pyridoxine) etc. Maize is also rich

			in phosphorous, zinc, copper, iron, magnesium, selenium and also has small amount of calcium and potassium. So maize is one of the good source of dietary fiber and protein which contains a very low fat and sodium (salt)
4.	Wheat /Gahum	*Triticum aestivum*	Whole wheat contains a variety of nutrients such as Vitamin B3, B2, B1, folic acid, copper, calcium, phosphorus, zinc, fiber, and iron, and removing out on them can have an impact on your diet. It contains catalytic elements, Vitamin E, Vitamin B, mineral salts, copper, calcium, iodide, magnesium, zinc, potassium, manganese, Sulphur, silicon, chlorine, and arsenic, which is why it is a great foundation for any diet.

APPENDIX-C

Composition and Preparation of different culture media for total viable count and yeast and mold count

1. Plate Count Agar (PCA)

Composition	Gms/L
Enzymatic Digest of Casein/tryptone	5.0
Yeast Extract	2.5
Glucose	1.0
Agar	15.0

Final pH 7.0 ± 0.2 at 25°C

Preparation of PCA as directed by company:

Suspend 23.5 grams in 1000 ml distilled water. Heat to boiling to dissolve the medium completely. Sterilize by autoclaving at 15 lbs. pressure (121°C) for 15 minutes. Cool to 45-50°C.Mix well and pour into sterile Petri plates.

*****Total Plate Number (ALT) is a number that indicates the number of mesophilic aerobic bacterial colonies present per gram or per milliliter of test sample. The principle of ALT is a method intended to calculate the growth of aerobic mesophyll bacteria colonies after the sample is planted on a solid media plate by pouring (poure plate) which is then incubated for 24-48 hours at 35-37°C.

2. Potato Dextrose Agar(PDA)

Potato Dextrose Agar (PDA) is used for the cultivation of fungi. Potato Dextrose Agar (PDA) is a general purpose medium for yeasts and molds that can be supplemented with acid or antibiotics to inhibit bacterial growth.

Composition	Gm/l
Dextrose	20 g
Potato extract	4 g*
Agar	15 g

*4.0gm of potato extract is equivalent to 200gm of potato infusion

If supplement added: tartaric acid – 1.4 gm

Final pH -3.5 +/- 0.3 at 25°C

Preparation of PDA as directed by company:

Suspend 39 grams of dehydrated media (supplied by commercial suppliers) in 1000 ml distilled water. Heat to boiling to dissolve the medium completely. Sterilize by autoclaving at 15 lbs. pressure (121°C) for 15 minutes. Mix well before dispensing.

*Culture Media were used from Hi-Media Company

APPENDIX- D

Sensory evaluation sheet for formulated gluten free cookies

Panelist I D: Date: ____________

Respected panelist, You are given 17 samples of cookies and taste them one at a time. Start with any cookie and take a bite, holding it in your mouth for a few seconds. Then, rate the taste of the cookie using the 9 point hedonic scale provided. After evaluating each cookie, rinse your mouth before trying the next sample.

Sample	Color	Apperance	Taste	Texture	Flavor	Crunchiness	Overall Acceptability
MBK							
MBY							
MABK							
MABY							
WBK							
WBY							
WABK							
WABY							
BWBK							
BWBY							
BWABK							
BWABY							
MzBK							
MzBY							
MzABK							
MzABY							
Control							

*****Color, flavor, taste, texture, crunchiness, Overall acceptability (OAC) is used as testing parameters for sensory evaluation

Hedonic Rating Scale

9. Like Extremely	4. DislikeSlightly
8. Like Very much	3. Dislike Moderately
7. Like Moderately	2. Dislike Very Much
6. Like Slightly	1. Dislike Extremely
5. Neither Like nor Dislike	

Here,

MBK=Millet cookies treated with baking powder,

MBY=Millet cookies treated with baker's yeast,

MABK=Millet cookies treated with amylase and baking powder, MABY=Millet cookies treated with amylase enzyme and baker's yeast, WBK=Whole grain wheat cookies treated with baking powder,

WBY= Whole grain wheat cookies treated with baker's yeast,

WABK= Whole grain wheat cookies treated with amylase enzyme and baking powder,

WABY= Whole grain wheat cookies treated with amylase enzyme and baker's yeast,

BWBK=Buckwheat cookies treated with baking powder,

BWBY= Buckwheat cookies treated with baker's yeast,

BWABK= Buckwheat cookies treated with amylase enzyme and baking powder,

BWABY= Buckwheat cookies treated with amylase enzyme and baker's yeast,

MzBK=Maize cookies treated with baking powder,

MzBY=Maize cookies treated with baker's yeast,

MzABK= Maize cookies treated with amylase enzyme and baking powder, MzABY= Maize cookies treated with amylase enzyme and baker's yeast

APPENDIX E

Procedure for the determination of moisture content and ash content of cookies

For Moisture Content:

By Hot air oven method: The sample was weighed and heated in an insulated oven to constant weight. The difference in weight is the water that has evaporated. The oven must be thermostatically controlled and usually set at 100°C or 105°C. 5 gram sample was weighed in aluminum moisture disc and then kept in 105°C for 4 hours. After that moisture content (%) was calculated using following formula:

$$\text{Moisture Content (\%)} = \frac{W1-W2}{W} \times 100\%$$

Where,

W = Wt. of sample

W1 = Wt. of aluminum moisture disc +sample before oven drying

W2 = Wt. of aluminum moisture disc +sample after hot air oven drying at 105°C for 4 hours

For Ash Conent:

By Dry Ashing method: Ash was determined by AOAC (1990) method. About 2 -5g of each sample was ignited at 560°C in a muffle furnace for 6 h. The residue was cooled in a desiccator and weighed. Total ash content was determined using following formula:

$$\text{Ash (\%) (Drybasis)} = \frac{MASH}{MDRY} \times 100\%$$

Where,

MASH = Mass of ash

MDRY = Sample or mass to be dried

Another formula can also use,

$$Ash\ (\%) = \frac{W1 - W2}{W} \times 100\%$$

Where, W1 = wt. of crucible +ash

W2 = wt. of crucible

W = wt. of sample

APPENDIX-F

Preparation of different chemical and reagents

1. **Preparation of Molisch's Reagent :**
   - 5% solution of α naphthol was added in 20 ml ethanol

2. **Preparation of Seliwanoff's Reagent:**
   - 50 mg of resorcinol, was weighed, then dissolved in 33ml of conc.HCl and diluted up to 100ml

3. **Preparation of Iodine Solution:**
   - 1gm of iodine (I_2) was weighed in breaker then 2gm of potassium iodide (KI) was weighed separately.
   - Little amount of potassium iodide (KI) was added into the beaker containing iodine and then added 1ml of distilled water ,stirred to dissolve.
   - This procedure was repeated till iodine has completely dissolved and made up the final volume (20ml) with water.

4. **Preparation of Bial's Reagent:**
   - 300gm of orcinol was weighed and then dissolved in 100ml of conc. HCl
   - 5 drops or 0.25mlof ferric chloride solution was added and mixed it well.

5. **Preparation of Fehling's Reagent:**
   - Fehling's solution was prepared freshly by mixing equal volume of Fehling's solution A (5ml) and Fehling's solution B (5ml).

6. **Preparation of Barfoed's Reagent:**
   - 6.65 gram of copper acetate was dissolved in 100ml of distilled water
   - Then 0.9 ml (900 µL) of glacial acetic acid was added on it

APPENDIX-G

Procedure for Microbiological assessment of cookies

1. **Total Plate Count:**

   General Steps: Standard Plate Count (By Pour Plate Method)

   1. Dilute sample to get a countable plate:

   - At first 10 gm. of cookies sample was weighed and diluted at 90ml sterile distilled water, dilution was made up to 10-3.

   2. Then 1ml diluted sample was added on a sterile petri dish

   3. After that culture media was poured on it; mixed gently; let it dry (Plate Count Agar preparation: 2.82gm of PCA was weighed and dissolve into distilled water, volume made up for 120ml.)

   4. When the media was solidified then incubated at 37 °C for 12-24hours

   5. Count plates: In this step, the numbers of viable organism were counted in the incubated plate. Colony forming unit per milliliter (CFU/ml) was calculated by using following formula:

$$\text{CFU/ml} = \frac{\text{No. of colonies on plate x dilution factor}}{\text{Volume of sample}}$$

2. **Yeast and Mold Count (YMC) :**

   - General Steps of Yeast and Mold Count(YMC)by spread plate technique:

   1. Sterile petriplates were taken and Potato Dextrose Agar (PDA) was poured and left it to solidfy.

   2. Serial dilution was done up to 10-3. At first dilution, 10 gm. of cookies sample was weighed and diluted at 90ml sterile distilled water (10-1), then take 1ml of the sample and transfer it to the second tube. Label it as 10-2 and so on.

3. **Spread plating:**

1. 0.1 ml diluent from the appropriate desired dilution series was pipette out and inoculated onto the center of the surface of an agar plate (PDA).

2. Then L-shaped glass spreader (hockey stick) was dipped into alcohol.

3. The glass spreader was flamed over a Bunsen burner. Then spread the sample evenly over the surface of agar using a cool alcohol-flamed glass rod spreader, carefully rotating the Petri dish underneath at an angle of 45° at the same time.

4. Incubate the plate at 37°C for 24-48 hours.

5. Count plates: In this step, the numbers of viable yeast and mold species present in the incubated plate were counted using given formula.

$$\text{CFU/ml} = \frac{\text{No. of colonies on plate x dilution factor}}{\text{Volume of sample}}$$

APPENDIX-H

Procedure for Qualitative test of carbohydrates in cookies

1 .Molisch's test:

a) 2ml of different cookie sample solutions were taken in different dry test tubes and labeled it well.
b) 2ml of distilled water was taken in another tube as control.
c) 2-3drops of molisch's reagent was added to the sample solution.
d) 1-2ml of conc.H2SO4 was added along the side of the tube .
e) Reddish-violet colored products was developed at the interface of two compounds.

7. Iodine's test:

a) 2ml of different test samples were taken in different dry test tubes and labeled it well.
b) 2ml of distilled water was taken in another tube as control.
c) 2-3drops of iodine solution was added in each test sample tube.
d) Blue-dark color was developed in each test sample.

8. Seliwanoff's test:

a) 1ml of cookies sample was taken in test tube.
b) 5ml of Seliwanoff's reagent was added on each sample test tube.
c) Then, heated to boil for 30 seconds, formation of red color in test sample indicates the presence of ketoses sugar.

9. Fehling's test:

a) 1ml of different cookies sample's solution were taken in different dry test tubes and labeled it well.
b) 1ml of freshly prepared Fehling's reagent was added in each tube.
c) After that the tubes were kept in boiling water bath and then observed for red precipitates.

10. Bial's test

a) 2ml of bial's reagent was taken in different tubes and labeled it well.
b) 4-5 drops of test solution was added in each reagent tubes.
c) Kept in water bath for boiling.
d) Development of green color was observed on the each tube.

*Development of green color indicates presence of pentose sugar in sample.

11. Barfoed’s test:

a) 2ml of different sample’s solution was taken in clean and dry test tube.
b) 2ml of barfoed’s reagent was added on it.
c) Then the tubes were kept in boiling water bath and noted the time until brick red precipitation was observed.

*Development of brick red precipitate within 3-5 minutes indicates reducing monosaccharaides.

*Development of brick red precipitate within 7-12 minutes indicates reducing disaccharides.

APPENDIX-I

Table 3: Formulation recipe for the preparation of Millet-taichin flour cookies (3:1)

Ingredients	MBK	MBY	MABK	MABY
Millet flour	375gm (75%)	375gm	375gm	375gm
Taichin flour	125gm (25%)	125gm	125gm	125gm
Icing sugar	200gm (40%)	200gm	200gm	200gm
Shortening	150gm (30%)	150gm	150gm	150gm
Butter	80gm (16%)	80gm	80gm	80gm
Sodium Bicarbonate	2.5gm (0.5%)	2.5gm	2.5gm	2.5gm
Ammonium Bicarbonate	2.5gm (0.5%)	2.5gm	2.5gm	2.5gm
Custard powder	10gm (2%)	10gm	10gm	10gm
Skim milk powder	20gm (4%)	20gm	20gm	20gm
Salt	5gm (1%)	5gm	5gm	5gm
Amylase enzyme	0gm	0gm	0.1gm	0.1gm
Baking powder	4gm (0.8%)	0gm	4gm	0gm
Baker's yeast	0gm	10.41gm(2.04%)	0gm	10.41gm
Butter flavor	4gm (0.8%)	4gm	4gm	4gm
Water	180gm	180gm	180gm	180gm

*Recipe for the formulation of millet cookies using baking powder (MBK)

*Recipe for the formulation of millet cookies using amylase enzyme and baking powder (MABK)

*Recipe for the formulation of millet cookies using baker's yeast (MBY)

*Recipe for the formulation of millet cookies using baker's yeast and amylase enzyme (MABY**)**

Table 4: Formulation recipe for the preparation of whole grain wheat-taichin flour cookies (3:1)

Ingredients	WBK	WBY	WABK	WABY
Whole grain wheat flour	375gm (75%)	375gm	375gm	375gm
Taichin flour	125gm (25%)	125gm	125gm	125gm
Icing sugar	200gm (40%)	200gm	200gm	200gm
Shortening	150gm (30%)	150gm	150gm	150gm
Butter	80gm (16%)	80gm	80gm	80gm
Sodium Bicarbonate	2.5gm (0.5%)	2.5gm	2.5gm	2.5gm
Ammoium Bicarbonate	2.5gm (0.5%)	2.5gm	2.5gm	2.5gm
Custard powder	10gm (2%)	10gm	10gm	10gm
Skim milk powder	20gm (4%)	20gm	20gm	20gm
Salt	5gm (1%)	5gm	5gm	5gm
Amylase enzyme	0gm	0gm	0.1gm	0.1gm
Baking powder	4gm (0.8%)	0gm	4gm	0gm
Baker's yeast	0gm	10.41gm	0gm	10.41gm (2.08%)
Butter flavor	4gm (0.8%)	4gm	4gm	4gm
Water	200gm (40%)	200gm	200gm	200gm

*Recipe for the formulation of whole grain wheat-taichin flours cookies using baking powder (WBK)*Recipe for the formulation of whole grain wheat-taichin flours cookies using baking powder and amylase enzyme (WABK)*Recipe for the formulation of whole grain wheat –taichin flours cookies using baker’s yeast (WBY)* Recipe for the formulation of whole grain wheat-taichin flours cookies using baker’s yeast and amylase enzyme (WABY)

Table 5: Formulation recipe for the preparation of whole grain buckwheat-taichin flour cookies (3:1)

Ingredients	BWBK	BWBY	BWABK	BWABY
Buck Wheat Flour	375gm (75%)	375gm	375gm	375gm
Taichin flour	125gm (25%)	125gm	125gm	125gm
Icing sugar	200gm (40%)	200gm	200gm	200gm
Shortening	150gm (30%)	150gm	150gm	150gm
Butter	80gm (16%)	80gm	80gm	80gm
Sodium Bicarbonate	2.5gm (0.5%)	2.5gm	2.5gm	2.5gm
Ammoium Bicarbonate	2.5gm (0.5%)	2.5gm	2.5gm	2.5gm
Custard powder	10gm (2%)	10gm	10gm	10gm
Skim milk powder	20gm (4%)	20gm	20gm	20gm
Salt	5gm (1%)	5gm	5gm	5gm
Amylase enzyme	0gm	0gm	0.11gm	0.11gm
Baking powder	4gm (0.8%)	0gm	4gm	0gm
Baker's yeast	0gm	10.41gm (2.04%)	0gm	10.41gm
Butter flavor	4gm (0.8%)	4gm	4gm	4gm
Water	190gm (38%)	220gm	203gm	203gm

*Recipe for the formulation of buckwheat-taichin flours cookies using baking powder (BWBK)

*Recipe for the formulation of buckwheat –taichin flours cookies using baking powder and amylase enzyme (BWABK)

*Recipe for the formulation of buckwheat-taichin flours cookies using Baker’s yeast (BWBY)

*Recipe for the formulation of buckwheat-taichin flours cookies using Baker's yeast and amylase enzyme (BWABY**)**

Table 6: Formulation recipe for the preparation of whole grain maize-taichin flour cookies (3:1)

Ingredients	**MZBK**		**MZBY**	**MZABK**	**MZABY**
Maize flour	375gm	(75%)	375gm	375gm	375gm
Taichin flour	125gm	(25%)	125gm	125gm	125gm
Icing sugar	200gm	(40%)	200gm	200gm	200gm
Shortening	150gm	(30%)	150gm	150gm	150gm
Butter	80gm	(16%)	80gm	80gm	80gm
Sodium Bicarbonate	2.5gm	(0.5%)	2.5gm	2.5gm	2.5gm
Ammoium Bicarbonate	2.5gm	(0.5%)	2.5gm	2.5gm	2.5gm
Custard powder	10gm	(2%)	10gm	10gm	10gm
Skim milk powder	20gm	(4%)	20gm	20gm	20gm
Salt	5gm	(1%)	5gm	5gm	5gm
Amylase enzyme	0gm		0gm	0.1gm	0.1gm
Baking powder	4gm	(0.8%)	0gm	4gm	0gm
Baker's yeast	0gm		10.41gm	0gm	10.41gm
Butter flavor	4gm	(0.8%)	4gm	4gm	4gm
Water	152gm	(30.4%)	152gm	152gm	152gm

- Recipe for the formulation of maize-taichin flours cookies using baking powder (MZBK)
- Recipe for the formulation of maize-taichin flours cookies using baking powder and amylase enzyme (MZABK)
- Recipe for the formulation of maize-taichin flours cookies using baker's yeast (MZBY)