Cold plasma treatment to improve the safety and quality of pearl millet flour

by

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Abstract

Pearl millet flour (PMF) has a well-balanced protein content with fat, gluten-free, and low glycemic index, making it a substitute diet for diabetes and celiac disease. However, PMF spoils quickly due to enzymatic rancidity, thus limiting its shelf life, and anti-nutrient factors, such as tannins and phytic acid, also limit its usage. This study aims to inactivate quality deteriorating enzymes, viz. lipase, lipoxygenase, polyphenol polyphenol oxidase (PPO), and peroxidase (POD) by using cold plasma (5-25 kV; 2-10 min). Cold plasma treatment at 25 kV/8 min, led to 95.9 %, 99.2 %, 94.8 %, and 100 % inactivation of lipase, lipoxygenase, peroxidase, and PPO activity, respectively. Cold plasma-induced enzyme inactivation followed n th-order kinetics, such as 1.55, 1.35, 1.15, and 1.05 for lipase, peroxidase, lipoxygenase, and PPO, respectively. Steam treatment at 100 °C/4 min inactivated lipase, lipoxygenase, peroxidase, and PPO by 97.9, 98.8, 97.6, and 100 %, respectively. The PMF showed 24.0 g/hg and 19.5 g/hg reduction in phytic acid and 77.7 g/hg and 16.9 g/hg reduction of tannin content at 25 kV/8 min of cold plasma treatment and steam treatment at 100 °C/5 min, respectively. Color, particle size, and proximate composition remained unaffected; a reduction in secondary oxidation products and minor modifications in the secondary structure of the protein were observed in both treatments.

Apart form the enzyme activity, PMF is contaminated with pathogenic species due to poor and unhygienic postharvest practices. This study aims to inactivate Salmonella spp, which cause foodborne illnesses, using the cold plasma treatment (20-28 kV/20-60 min). The effectiveness of the treatment is observed through different combinations of treatments, such as the contaminated pearl millet grains are treated with cold plasma treatment (A), grains tempered with plasma-activated water (B), and the contaminated pearl millet grain is milled to flour and treated with cold plasma treatment (C). Furthermore, with cold plasma treatment as all possible combinations

of treatment such as A+B, B+C, A+C, and A+B+C. Cold plasma induced the highest microbial inactivation of 3.0 log cycle, with the combination treatment of A+B+C such as the cold plasma treatment of the grain, followed by tempering with plasma-activated water and cold plasma treatment of the flour at 28 kV/60 min. Through the individual treatment of the grains (A) and the combination of A + C achieved the highest reduction of 1.4 and 2.6 log CFU/g at 28 kV/60 min, respectively. A+B+C treatment condition shows a decrease in true, bulk, tapped density, an increase in the geometric mean diameter, and changes in the color profile observed due to the tempering of the grains.

The third objective of the study is to optimizing its milling methods through pin mill and hammer mill and determination of dough rheological properties. Furthermore, develop bread using cold plasma-treated tempered and untempered pearl millet flour (PMF) using the optimal mixture design. Pin-milled PMF has a uniform particle distribution, and tempered pearl millet flour exhibited the highest yield, 93.5%. Varied proportions of the wheat flour (70-87 g), pearl millet flour (10-27 g), and shortening agent (3-6 g) were used to assess the bread hardness, crust color, and specific volume, as per D-optimal mixture design. A special cubic model was fitted to each response in both conditions. An optimized blend for the tempered flour composed of 85.5 g refined wheat flour,11.5 g PMF, and 3 g of shortening, having the hardness, total color change of crust, and specific volume 6.06 N, 5.47, and 2.82 $mL.g^{-1,}$ respectively. Similarly, the untempered pearl millet flour has 75.6 g wheat flour, 18.3 g PMF, and 6 g of the shortening agent, comprising the hardness, total color change of crust, and specific volume 7.32 N, 6.60, and 3.04 $mL.g^{-1}$ respectively. Cold plasma treatment 25 kV/8 min enhances the overall functional properties and texture attributes of the bread.

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Chapter 1 - Introduction

Pearl millet (*Pennisetum glaucum* (L.) R. Br.) is a climate-resilient cereal widely cultivated in arid and semi-arid regions due to its ability to thrive in poor soils and drought-prone areas, making it a crucial crop for food security (Meena et al. 2024; Satyavathi et al. 2021). This millet is gaining attention due to its properties and alignment with nutritional and sustainable development goals (Boukid et al. 2018). Pearl millet is grown on over 30 million hectares worldwide, mainly in Asia (>10 million hectares) and Africa (~18 million hectares), with India leading production at 38.4% (APEDA, 2022). Recognized for its high energy value, quality proteins, dietary fiber, and rich micronutrient content, it serves as an affordable nutritional source for millions, particularly in Asia and Africa (Krishnan and Meera 2018; Saleh et al. 2013; Seal et al. 2021)

1.1. Problem Statement

Despite its nutritional benefits, pearl millet flour (PMF) has a short shelf life (5–7 days) due to rapid rancidity caused by lipolytic enzymes, primarily lipase, which hydrolyzes glycerides into free fatty acids (FFAs), leading to bitterness and off-flavors (Padmaja et al. 2023). With a fat content of 3%–8%, PMF undergoes both enzymatic and oxidative rancidity. Enzymatic rancidity is driven by lipase, lipoxygenase (LOX), polyphenol oxidase (PPO), and peroxidase (POD), whereas oxidative reactions involving unsaturated fatty acids contribute to non-enzymatic rancidity. Additionally, antinutrients such as polyphenols, tannins, and phytic acid in pearl millet further restrict the bioavailability of essential minerals (Singh et al. 2015). Given the increasing global demand for gluten-free and fiber-rich foods, enhancing the stability and shelf life of PMF is essential for its broader adoption in commercial food products.

Various processing strategies have been explored to extend the shelf-life of PMF by reducing rancidity and improving its stability. These methods can be broadly categorized into biological

(fermentation, malting, and germination), mechanical (decortication or pearling), thermal, and nonthermal approaches. Fermentation is an effective biological preservation method that enhances both the nutritional value and shelf-life of PMF. Microorganisms such as lactic acid bacteria and yeast generate organic acids and antimicrobial compounds, which naturally inhibit spoilage organisms and reduce antinutrients. Similarly, malting and germination stimulate enzymatic activity in sprouted seeds, breaking carbohydrates, proteins, and lipids into simpler compounds, thereby improving digestibility (Akinola et al. 2017). Beyond biological methods, a mechanical process, pearling, which removes the bran layer, enhances the shelf-life of PMF by reducing lipid oxidation. However, this process also leads to nutrient loss, particularly of iron, zinc, and antioxidants, which are primarily concentrated in the grain's outer layers.To counteract the lipid degradation and prevent rancidity, thermal treatments are widely applied to PMF flour. Roasting, blanching, hydrothermal processing, dry heating, and steaming effectively reduce FFA formation by inactivating lipase. However, extended heat exposure can modify sensory properties like color and texture (Goyal and Chugh 2017). Blanching, for example, involves brief boiling (98 °C for 30 s) followed by drying, effectively reducing enzyme activity (Pawase et al. 2021). Additionally, dry heating significantly inhibits lipase activity, minimizing lipid degradation during storage (Sankar et al. 2008). However, heating may also activate pro-oxidants, leading to oxidative degradation of triglycerides and a potential loss of antioxidants. Nonthermal techniques, including cold plasma, ultrasound, and irradiation, offer alternative methods to preserve PMF without heat exposure. Cold plasma generates reactive ions and free radicals that inactivate microorganisms and enzymes (Chakraborty et al. 2024). Ultrasound utilizes high-frequency soundwaves to induce physical and chemical changes, enhancing microbial safety and enzyme stability (Ma et al. 2023). Irradiation uses controlled doses of ionizing radiation to eliminate pathogens and reduce spoilage without

compromising the nutritional integrity of the flour (Sruthi and Rao 2021). These nonthermal techniques can alter the tertiary and quaternary structural arrangements of enzymes. Unlike thermal treatments, these methods do not induce color changes and maintain water holding capacity and gel formation, which are crucial for flour functionality (Sruthi and Rao 2021). Several studies have examined the impact of different processing techniques on the functional, nutritional, and storage properties of PMF, yet there remain gaps in understanding the specific role of enzymatic spoilage mechanisms. For instance, Kindiki et al. (2015) summarized the effect of various processing techniques, such as fermentation, germination, and roasting, on the sensory and nutritional properties of PMF. However, the influence of these processing conditions on enzyme activity that could lead to product deterioration was not addressed. Akinola et al. (2017) investigated the effects of various treatments like bioprocesses (fermentation and malting), physical debranning, and hydrothermal treatment (blanching), on the morphology, microbial decontamination, and functional attributes of PMF for potential industrial use. Yadav et al. (2012) explored the pasting properties of PMF after hydrothermal treatment. Kapoor and Kapoor (1990) studied the storage stability of PMF when treated with antioxidants, heat, defatting, and salting for 30 days. More recent reviews have provided comprehensive insights into storage stability, nutritional value, and antinutrient reduction in processed PMF. Sruthi and Rao (2021), for example, evaluated how different processing techniques influence the storage behavior of millet flours, including PMF. Rai et al. (2008) studied the various processing methods and their effects on pearl millet's nutritional and antinutrient profiles. Suma and Urooj (2017) reviewed the nutritional quality of pearl millet post-household processing methods. Another review by Ramasamy et al. (2024) summarized the effects of several processing techniques, such as milling, dehulling, malting, fermentation, blanching, acid treatment, and heating, on nutrient composition,

antinutritional factors, and product development. Goswami et al. (2023) investigated the influence of bioprocesses (malting, germination, and fermentation), decortication, and hydrothermal and dry heat (near-infrared [NIR], microwave, and ohmic heating) treatments on the shelf-life of PMF. Meena et al. (2024) summarized the impact of various processing methods on nutritional attributes and antinutrients in pearl millet. Although these studies provide valuable insights, most focus primarily on sensory, nutritional, or microbial parameters. There is limited emphasis on how processing conditions specifically modulate enzymatic activities—particularly lipase and other oxidative enzymes—that drive spoilage and the processing conditions to enhance the microbial safety in PMF. Apart from that there is limited research available on optimizing the bread formulation made with the PMF. This gap underscores the need for targeted studies exploring the relationship between processing techniques on enzymatic, microbial stability while emphasizing product development.

1.2. Research hypothesis

This study hypothesizes that cold plasma is an effective treatment to inactivate the spoilage-causing enzymes and foodborne pathogens and thus further enhance the dough and baking properties of the pearl millet flour while preserving the nutritional and functional properties of the flour.

1.3. Research objectives

This research focused on understanding the effect of the cold plasma treatment on the safety and quality aspects of the pearl millet flour as a potential alternative to thermal treatment. Specific objectives of this study are listed below.

1. To analyze the impact of cold plasma treatments on enzyme inactivation kinetics and their effects on the physicochemical properties of pearl millet flour.

2. To determine the efficiency of the cold plasma treatment as an intervention strategy to achieve microbial safety in pearl millet flour.

3. To study the influence of cold plasma exposure on the functional and baking properties of the pearl millet flour.

1.4. Research outline

The thesis consists of six chapters, each of which contributes to the understanding of this study. Chapter 1: provides an overview of the study's objectives and presents the problem statement. Chapter 2: provides a comprehensive literature review of the pearl millet varieties, nutritional and functional properties, and different processing techniques of pearl millet flour. Chapter 3: provides the effects of cold plasma and stream treatment on the enzyme inactivation of the pearl millet flour and its influence on the physicochemical and antinutrient properties. Chapter 4: provides the effect of the cold plasma treatment to improve the safety of the pearl millet flour. Chapter 5: provides the influence of the cold plasma treatment on the dough and baking properties of the pearl millet flour. Chapter 6: provides the lastly summary of findings, conclusion, and recommendations for future work based on the presented studies.

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Chapter 2 - Literature Review

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2.1. Pearl Millet Grain Anatomy

Pearl millet is a caryopsis-type grain with an ovoid or globose shape, typically gray, white, yellow, brown, or purple (Figure 2.1). The grain consists of 8.4% pericarp, 75% endosperm, and 16.5% germ, with a 4.5:1 ratio of endosperm to germ. The pericarp is made up of three layers: the epicarp (outer), mesocarp (middle), and endocarp (inner), with the mesocarp lacking starch granules. During milling, the pericarp breaks at the cross and tube cell layers. Beneath the endocarp lies the seed coat, and the endosperm is the largest part of the kernel, composed of an aleurone layer (rich in minerals, B vitamins, and oil) and two zones: corneous (dense with starch granules) and floury (contains more starch and protein bodies and is more amenable to enzyme digestion). The starch granules in the floury endosperm are spherical and larger than those in the corneous zone. The germ in pearl millet is larger than in sorghum and other millets (Serna-Saldivar and Espinosa-Ramírez 2019). The germ comprises the embryonic axis and scutellum, rich in lipids, proteins, enzymes, and minerals.

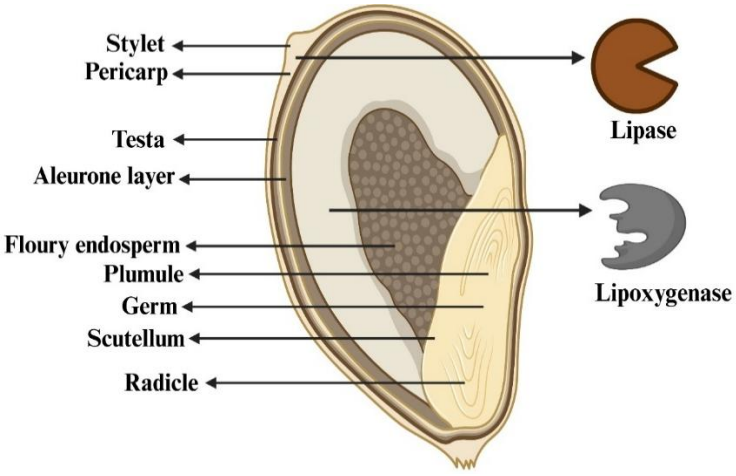

Figure 2.1: Anatomy of the pearl millet grain. Redrawn from Source: Hassan et al. (2021)

2.1.1. Nutritional Composition

Geographically, Indian varieties of pearl millet are cultivated across Maharashtra and Haryana under warm climates and rain-fed or irrigated conditions. Sudanese varieties, such as IS 91777, thrive in rain-fed and arid soils, indicating adaptability to different environments. However, variations in cultivation conditions influence nutritional composition, making some varieties more suitable for specific dietary or processing needs. Pearl millet contains 6.5% (HC10) to 12.4% (Dhanshakti) moisture, 1.6%–3.1% ash, 2.7%–7.1% fat, 8.5% (IS 880004) to 15.1% (Kabti) protein, 1.2% (HC20) to 4.0% (Kabti) crude fiber, and 58.5% (Kabti) to 72.5% (HC10) carbohydrates, depending on the genotype (Table 2.1). Mineral content also differs significantly, with calcium ranging from 100 mg/kg (IS 91666) to 800 mg/kg (IS 91777) and phosphorus showing considerable variation, with IS 91333 having the highest at 990 mg/kg. Zinc and iron levels also fluctuate, with IS 89111 showing the highest iron content (180 mg/kg). Pearl millet is a high-energy cereal with beneficial unsaturated fats, including omega-3s, which support heart health by lowering cholesterol (Pei et al. 2022). This remarkable millet features a well-balanced essential amino acid profile, boasting high concentrations of threonine, tryptophan, and leucine compared to other cereals (Lokeswari et al. 2021). It is gluten-free, and its carbohydrates have a lower glycemic index (54–68) and higher resistant starch (8%–12%) and dietary fiber (11%–13%) (Nambiar et al. 2011; Pei et al. 2022; Kaimal et al. 2021). Being glutenfree, pearl millet is an ideal substitute for individuals with celiac disease. Its high fiber content aids in digestion, helps regulate blood sugar levels, and promotes heart health. The primary components of pearl millet grain protein include both essential and non-essential amino acids, with leucine (1.70%), phenylalanine (0.71%), threonine (0.52%), isoleucine (0.58%), glutamic acid (3.58%), aspartic acid (1.32%), proline (1.07%), and alanine (1.33%) contributing to the total amino acid profile of pearl millet

protein (Kapustin et al. 2023). Because it has a low glycemic index, it can be used as a substitute diet to help manage weight and reduce the chance of developing long-term conditions like diabetes (Dias-Martins et al. 2019).

2.1.2. Functional Properties

PMF has desirable functional properties such as water absorption capacity (WAC), oil absorption capacity (OAC), foaming capacity, and emulsion capacity, which vary with the genotypes (Table 2.2). These properties are crucial for different food applications. WAC varies across varieties, ranging from 1.53 mL/g (W445) to 1.86 mL/g (Phule Adishakti), indicating differences in hydration potential, influencing dough formation and texture. OAC, an important factor for flavor retention and texture, also shows variation, with Phule Adishakti exhibiting the highest OAC (1.57 mL/g), whereas HC10 has the lowest (1.05 mL/g). Foaming capacity, which impacts the aeration properties of flour, has been reported for various varieties. W445 demonstrated the highest foaming capacity (0.25 mL/mL). Emulsion capacity, an essential property for stabilizing O/W emulsions in food formulations, ranges from 0.45 mL/mL (HC10) to 0.75 g/g (Phule Mahashakti), suggesting that some varieties may be more suitable for processed food applications requiring emulsification

Table 2.1: Proximate and nutrient compositions of various pearl millet varieties.

Variety	Proximate component (%)						Minerals (g/kg)				Antinutrients (g/kg)		Majorly cultivated in			Reference
	Moisture	Ash	Fat	Protein	Fiber	CHY	Ca	P	Zn	Fe	Phytate	Tannins	Country	Region	Conditions	
Shanti	11.7	2.1	5.9	11.5	2.2	67.1	0.413	2.967		0.064	5.963	2.298	India	Maharashtra	Warm climate, irrigated, and rain-fed areas	Kulthe et al. (2016)
Dhanshakti	12.4	2.7	5.4	11.6	2.6	66.4	0.426	3.278		0.081	5.666	2.327				
Pioneer 86M64	11.2	2.0	5.1	10.9	2.0	68.8	0.400	2.556		0.050	6.188	2.256				
WHC901	7.8	1.8	6.1	11.8	1.2		0.509		0.0029	0.068			India	Haryana	Warm climate, and rain-fed areas	Jandu & Kawatra (2019)
HC20	7.4	1.9	6.1	11.9	1.2		0.517		0.043	0.072						
HHB67 Imp	8.6	1.7	5.8	12.4	1.6		0.548		0.031	0.064			India	Haryana	Warm climate, and rain-fed areas	Goyal & Chugh (2017)
Phule Adishakti	10.5	2.6	5.2	12.1	2.0	67.4							India	Maharashtra	Warm climate, irrigated, and rain-fed areas	Pawase et al. (2021)
Dhanshakti	11.0	2.4	5.5	11.6	1.5	67.7										
Phule Mahashkti	11.6	2.6	5.3	11.1	1.6	66.5										
Pioneer 86M32	11.6	2.2	4.9	10.4	1.8	68.8										
HC20	11.5	3.1	4.8	10.8	1.7	66.9										
IS91777		1.9	2.7	9.1	3.8	70	0.800	8.80	0.060	0.070	6.180		Sudan	Shambat	Rain-fed areas and arid soils	Abdalla et al. (1998)
IS91666		2.0	7.1	9.3	3.8	67.3	0.100	7.00	0.060	0.100	5.30					
IS91333		2.4	4.6	12.1	3.9	61.0	0.400	9.90	0.060	0.140	7.950					
IS89111		1.9	5.6	10.1	2.6	66.2	0.500	5.00	0.065	0.180	4.420					
IS880004		2.2	5.9	8.5	3.7	67	0.500	9.90	0.053	0.110	7.960					
IS833		1.8	6.9	10.4	3.0	66.6	0.700	4.50	0.060	0.100	3.540					
IS843		1.8	5.2	11.0	3.7	65.2	0.500	4.90	0.053	0.090	3.540					
Kabti		1.9	4.7	15.1	4.0	58.5	0.400	4.90	0.060	0.100	3.550					
YD-X3		1.6	4.8	12.8	3.7	61.1	0.500	5.50	0.060	0.100	4.430					
Tihama		2.0	4.8	13.7	3.9	60.4	0.500	4.90	0.070	0.140	3.540					
HC10	6.5	1.6	6.5	9.7	3.1	72.5							India	Haryana	Warm climate and rain-fed areas	Siroha et al. (2016)
HHB67	6.9	1.6	6.6	9.9	3.4	71.5										
W445	7.6	1.9	5.1	11.3	3.2	70.9										
GHB732	7.7	1.9	5.6	10.7	3.8	70.3										
HHB223	6.8	1.6	5.7	10.6	2.9	71.6										
HHB226	7.6	1.7	5.2	10.4	3.5	69.6							India	Haryana	Warm climate and rain-fed areas	Goyal & Chugh (2017)

The cell with no data means that the corresponding information is not available in the literature.

Table 2.2 Functional properties of various varieties of pearl millet.

Pearl millet variety	Water absorption capacity (mL/g)	Oil absorption capacity (mL/g)	Foaming capacity (mL/mL)	Emulsion capacity	Reference
HC10	1.73	1.05	0.18	0.45 mL/mL	Siroha et al. (2016)
HHB67	1.66	1.04	0.22	0.52 mL/mL	
HHB223	1.65	1.12	0.24	0.60 mL/mL	
HHB226	1.66	1.24	0.20	0.55 mL/mL	
W445	1.53	1.13	0.25	0.60 mL/mL	
GHB732	1.77	1.16	0.21	0.55 mL/mL	
Phule Adishakti	1.86	1.57		0.65 g/g	Pawase et al. (2021)
Dhanshakti	1.54	1.30		0.55 g/g	
Phule Mahashakti	1.58	1.36		0.75 g/g	
Pioneer 86M32	1.69	1.44		0.58 g/g	
HC20	1.67	1.23		0.50 g/g	

2.1.3. Color profile

The color profile of various pearl millet varieties indicates significant variations in lightness (L*), redness (a*), and yellowness (b*), which are influenced by pigment composition, including carotenoids and polyphenols (Table 2.3). Polyphenol compounds contribute to the color of pearl millet grain, which is present in the pericarp, germ, and aleurone layer of the grain. Phenolic compounds, such as ferulic acid and p-coumaric acid, are dominant contributors to pigmentation. Dark-colored millets have a higher phenolic content compared to lighter colored varieties (Nambiar et al. 2011; Triki et al. 2022). The Shanti, Dhanshakti, and Pioneer 86M64 varieties exhibit the highest lightness (L*>95) and the lowest yellowness (b* < 2.22), making them visibly lighter and less yellow compared to other varieties. Conversely, HC10, HHB67, HHB223, HHB226, W445, and GHB732 varieties have lower L* values (75.1–78.4) and higher b* values (9.9–13.5), giving them a darker and more yellow appearance. The chroma (C*) and hue (H*) values further support these distinctions, with darker varieties generally showing higher chroma

values, indicating more intense coloration. The presence of carotenoids contributes to the yellow hue, whereas polyphenols help protect against oxidation, which is crucial for delaying rancidity in PMF.

Table 2.3 Color profile of various varieties of pearl millet.

Variety	*L**	*a**	*b**	*C**	*H**	Reference
Shanti	95.69	-0.10	2.22	2.22	92.73	Kulthe et al. (2016)
Dhanshakti	95.61	-0.11	1.91	1.92	93.53	Kulthe et al. (2016)
Pioneer 86M64	95.64	-0.16	1.99	2.00	94.78	Kulthe et al. (2016)
HC-10	75.1	0.72	10.6	10.62	86.11	Siroha et al. (2016)
HHB-67	76.0	0.43	10.4	10.40	87.63	
HHB-223	78.4	0.32	9.9	9.90	88.14	
HHB-226	77.8	0.59	10.9	10.91	86.90	
W-445	77.5	1.64	13.5	13.60	83.07	
GHB-732	75.4	0.74	11.5	11.53	86.31	

2.1.4. Phytochemicals

The phenolic profile of pearl millet plays a key role in its antioxidant activity. Among the genotypes, GHB732 exhibits the highest antioxidant activity (46.7%) and total phenolic content (3137 mg GAE/kg), indicating its strong antioxidant potential (Table 2.4). HHB67 improved and showed a total polyphenolics of 2750 mg GAE/kg. W445 has the highest flavonoid content (2406 mg CE/kg), suggesting it as a rich source of flavonoids. HC10 shows the highest metal-chelation activity (20.3%), which may help prevent oxidative stress caused by metal ions. Its antioxidant properties support overall health by reducing oxidative stress (Xiong et al. 2019). Pearl millet's fibrous pericarp is rich in inhibitory factors like phytic acid, polyphenols, and fiber. The polyphenol range is between 1.55 and 4.45 g/kg. The bran portion of pearl millet has a significant level of polyphenols compared to the endosperm fraction. It contains lower levels of tannins than sorghum, with 0.61 mg/g in pearl millet versus 0.79 mg/g in sorghum. Pearl millet also contains

3.54–8.92 mg/g of phytic acid, whereas sorghum has a higher concentration at 18.37 mg/g (Sharma and Garg 2023). Although these levels are lower than sorghum, both grains contain antinutritional factors that can impact nutrient bioavailability. The endosperm contains less phytic acid than the aleurone layer. Multivalent metal ions like calcium and iron bind with phytates, thus inhibiting their absorption in the body (Saleh et al. 2013). Conversely, phytate's ability to bind pro-oxidant cations like iron and copper may enhance the stability of PMF triglycerides. However, this chelation of dietary minerals in the gastrointestinal tract reduces their bioaccessibility (Dendegh et al. 2019). It is rich in proteins that can be a source of bioactive peptides that play an important role in preventing chronic diseases (Cavazos and Gonzalez de Mejia 2013).

Table 2.4 Some phytochemical properties of various pearl millet genotypes.

Variety	**Antioxidant activity (%)**	**Total phenolic content (mg GAE/kg)**	**Total flavonoid content (mg CE/kg)**	**Metal chelating activity (%)**	**Reference**
HC10	37.7	2668	2484	20.3	Siroha et al. (2016)
HHB67	31.8	2555	2345	12.2	
HHB223	34.4	2394	1721	15.3	
HHB226	35.7	2678	1800	8.5	
W445	33.4	2404	2406	6.7	
GHB732	46.7	3137	2036	16.9	
PC 433	47.8	1760			Goswami et al. (2024)
HHB67-Imp	34.4	1730			
GHB732	49.5	1520			
HHB226	54.8	1930			
Dhanshakti	39.5	1530			
MPMH17	42.8	2070			
86M01	39.1	1570			
GHB744	47.5	1680			

2.1.5. Micronutrients

Pearl millet can serve as a dietary source of selenium, an essential trace element important for various physiological functions such as antioxidant activity, immune system regulation, and protection against infections (Xie et al. 2021). Potassium and phosphorus levels in pearl millet grain are higher than in other cereals. However, copper, magnesium, and sodium concentrations are similar to wheat (Krishnan and Meera 2018). Pearl millet is naturally rich in iron and zinc and offers solutions to combat mineral malnutrition through biofortification solutions. Enhancing this mineral's bioavailability will help address global hunger and malnutrition (Shahzad et al. 2014). The water-soluble vitamins riboflavin, niacin, thiamine, and tryptophan are abundant in pearl millet grain. It was claimed that the vitamin A content in this grain is 220 IU/100 g. The total carotenoid and beta-carotene content in pearl millet is reported to be 4.4 and 0.85 mg/kg, respectively (Sihag et al. 2015; Sachan and Shah 2023).

2.2. Use in Traditional Foods

PMF is used in many traditional food formulations, including unleavened bread (roti), fermented dishes (kisra and jalebies), and thin and thick porridges (toh). In Namibia, PMF is used to produce thick porridge (oshifima), thin porridge (etete), pancakes (omungome), cake (oshikwila), and beverages (oshikundu). Table 2.5 explains the procedure for making traditional PMF-based products. In Kenya, PMF is combined with a range of other cereal flours, such as finger millet, sorghum, and wheat, in commercial flour products to make uji (thin porridge) with a souring agent, a fruit acid. This product is based on thiakri, a traditional soured custard-like product from the Sahel region (Taylor et al. 2010). There are various kinds of traditional foods made with the PMF. Thick porridges or pastes called "tuwo" in West Africa, made by mixing PMF and water and heating for 10 min, typically solidify to the point where they can be easily divided into manageable

pieces for dipping into stew or sauce. Roti, a flat unleavened bread, is commonly made from PMF and other grains like sorghum and maize on the Indian subcontinent. “Couscous” or “arraw” is a steamed dish created by gelatinizing PMF with additives like sugar and okra gums. Idli is a steamed cake made from a fermented mixture of pearl millet and legume flour, often enjoyed at breakfast in India (Basak et al. 2023). Fura is created by grinding pearl millet into flour, rolling it into large balls, parboiling it, and blending it into a watery paste with fermented milk.

Table 2.5: Traditional products developed using pearl millet flour.

Local Name	Formulation	Reference
Kisra, Galettes, Dosai (fermented dishes)	In West Africa, it's called *Galettes*; in Sudan and Ethiopia, *Kisra*; and in India, *Dosai*. The flour is mixed with water and a starter, fermented for 12–24 h, then fried. For *Kisra*, water is added before cooking to create a runny paste for easy spreading. For *Dosai*, 1/3 of bean flour is added before fermentation.	Taylor & Duodu (2019)
Oshifima (thick porridge)	Oshifimá, a thick porridge, is prepared by boiling PMF in double the amount of water and stirring gradually. The mixture is cooked for 15-20 min till it becomes thick.	Devos & Ricquier (2022)
Koko	Koko, a millet porridge, is enjoyed as a snack. Pearl millet is soaked overnight, wet-milled with spices, and mixed with water to form a slurry. After sieving and fermentation, the mixture settles. The top water is boiled, and the sediment is stirred in to achieve the desired consistency.	Ilango & Antony (2021)
Tchobal	Tchobal is a fermented and gelatinized pearl millet dish. After dehulling, the grains are ground with water and steam-cooked into gelatinized balls. These are fermented for 24 h and served with dairy products or juice.	Adebiyi et al. (2018)
Boza	In Egypt, boza is a thick, pale yellow drink brewed from pearl millet with a tangy, acid-alcoholic aroma. Millet grains are soaked overnight and then cooked in 4-times water until soft. The mixture is blended with 1 cup sugar and strained into a thick liquid. Once lukewarm, 1/4 teaspoon of dry yeast is added and fermented for 1-2	Adebiyi et al. (2018)

	days at room temperature, with occasional stirring. It is served after fermentation.	
Bensaalga	Millet–groundnut blends are used to make a gruel by soaking pearl millet and groundnut in a 3:1 ratio for 16 h. The wet grains are milled with ginger and mint (2:1 ratio) added to 100 g of grains. The mixture is kneaded, sieved, and fermented for 24 h, then cooked for 5 min.	Ilango & Antony (2021)
Degue (tchobal)	Degue (tchobal) is a popular fermented and gelatinized pearl millet dish in Burkina Faso. The dehulled grains are ground with water and steam-cooked into gelatinized balls. These balls are fermented for 24 h and served with dairy products or juice.	Adebiyi et al. (2018)
Oshikundu	Oshikundu is made from a mixture of sorghum and pearl millet flours, and pearl millet bran. Hot water is mixed with PMF; then warm water is added along with sorghum malt and stirred again. After cooling, sorghum malt and bran are mixed in. Cold water is added with previously fermented Oshikundu for backslopping. The mixture is fermented for 46 h and served within 6 h.	Misihairabgwi & Cheikhyousse f (2017)
Omalodu (Alcoholic fermented beverages)	It is a traditional beer made from sorghum flour and PMF. The sorghum malt is mixed with cold water and boiled for 2 h. The cold and sieved mixture is fermented for 6-24 hours inside a pot called oshitoo. The mixture is fermented for 1 more hour with a small PMF and served afterwards.	Misihairabgwi&Cheikhyoussef. (2017)
Okatokele (beer)	A mixture of PMF, sugar, and water is stirred thoroughly and fermented in plastic buckets at room temperature for 8 to 24 h. The fermented mix is served as a ready-to-drink beer.	Misihairabgwi & Cheikhyousse f. (2017)
Epwaka (fermented beverage)	The pearl millet bran is mixed with water and sugar. The mixture is fermented at room temperature for 8 to 24 h. The fermented mix is served as a ready-to-drink beverage.	Misihairabgwi & Cheikhyousse f. (2017)
Tuwo (thick porridge)	A portion of PMF is cooked to form a thin, gelatinized liquid. The remaining flour is then added and cooked further to achieve the desired consistency. Variations can include alkaline or acidic	Adegbola et al. (2023)

	recipes, with some requiring overnight soaking in sour milk or brief fermentation before cooking.	
Couscous or arraw	It is made by gelatinizing and agglomerating PMF, with sugar and okra gum.	Hayes et al. (2020)
Idli	It is a popular breakfast in India. A mixture of pearl millet and legume flour is fermented overnight and steamed before serving.	Basak et al. (2021)
Fura	The mixture of PMF, water, and spices is compressed into dough balls and cooked in steam.	Adebiyi et al. (2018)
Roti	The dough is made of PMF with hot or cold water. In some regions, jaggery water, milk, or sugarcane juice is used. The dough is kneaded and shaped into balls and then flattened into discs.	Ali et al. (2023)
Debra, Thepla	Wheat flour is combined with PMF, and spices are added before making discs. The rotis, thus made, are called Debra or Thepla in Gujarat, India.	Basak et al. (2021)
Kurdigi, sandigai (papad)	This snack is prepared by creating a thick or thin spicy porridge with PMF. The mixture is either extruded using molds or spread directly on cloth and sun-dried. After drying, the snacks are fried in oil to achieve a crispy texture for consumption.	Kalange et al. (2020)
Kunu (non-alcoholic fermented beverage)	The pearl millet grains are coarsely ground to flour. The paste of PMF and hot water is fermented for several days. It is often served with ginger with an improved flavor.	Jideani et al. (2021)
Puri	The ground pearl millet grain is flattened and deep-fried to make puris.	Gangale & Jadhao (2016)
Khichdi	A mixture of pearl millet grains and split pulses is cooked in water to make khichri.	Basak et al. (2021)
Porridges	In Asia, PMF is soaked with sour milk and fermented overnight before cooking. In Africa, the pearl millet grains are steeped in water for 2-3 days. The steeped grains are crushed and cooked after bran removal.	Meena et al. (2024)
Flatbreads	For fermented bread, PMF is mixed with water and a starter, allowed to ferment for 12 to 24 h, and then cooked. In Nigeria, pearl millet is dry-milled into flour and combined with wheat flour for bread production. Chapati is prepared using PMF enriched with soybean flour.	Basak et al. (2021)

Thin porridges or gruels	Whole pearl millet grains are steeped in water for 2 to 3 days to initiate germination. After fermentation, the mixture is decanted and cooked.	Diarra et al. (2022)

Abbreviation: PMF, pearl millet flour.

2.3. Pearl Millet Flour

PMF is whole-grain flour. It has a fibrous seed covering, giving a gray-to-yellow look to PMF flour. The pericarp, aleurone, and endosperm are rich in polyphenolic pigments, and these result in a gray color in the flour (Ramalingam et al. 2024). It is obtained by passing through the mills several times, with the milling process varying by location. Several kinds of milling methods are employed on pearl millet, such as hand pounding, mortar and pestle, hammer milling, roller milling, and non-motorized grain milling. For decortication and hulling, machines such as rice hulling machines, rice hullers with polishers, abrasive mills, disks with mechanical dehullers, mechanical abrasive decorticators, rice polishers, and centrifugal shellers are used to remove the outer layers of grains. Figure 2.2 explains the process of manufacturing PMF from the grains. The PMF-making process begins with cleaning the grains through sieving. Next, the grains are conditioned with water in a 3:10 ratio to increase their moisture content, preparing them for milling. In some cases, the bran is separated using mechanical abrasive decortication, but this step is not always needed, as the whole PMF is preferred in some situations. Afterward, the grains pass through the first break roller milling. The flour fraction obtained through the first break roller milling is passed through the sifting and purification fraction. Pearl millet grain is milled through repeated passes to achieve the desired particle size smaller than 212 μm, which is classified as the first break flour. The remaining flour continues through the reduction roller milling process, where it is further milled and then again sifted and purified. The flour from the first and second fractions is then combined. In many traditional and household applications, whole-grain PMF is commonly

used due to its higher nutritional content and minimal processing requirements (Barrion 2008). Notably, pearl millet has a substantial germ portion, constituting 17.4% of the total kernel, surpassing other grains; thus, it comes naturally into the flour (Akinola et al. 2017). It does not have any polishing or refining steps. This flour has a strong taste, a short shelf-life due to enzymatic reactions, and a rotten flavor, resulting in low market appeal. This is one of the reasons rice and wheat eaters dislike it (Kulthe et al. 2016).

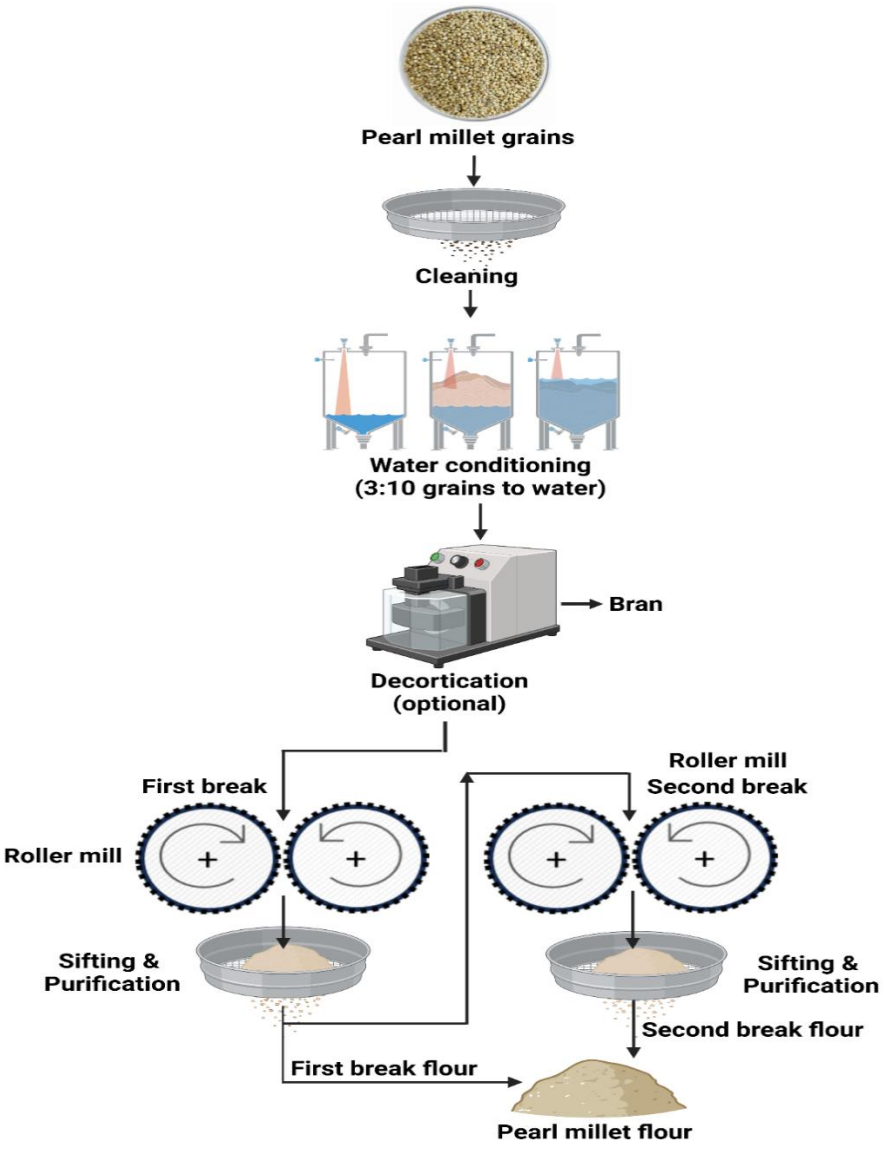

Figure 2.2: Flow chart showing the procedure to obtain flour from pearl millet grains.

2.4. Factors Influencing the Shelf-Life of PMF

Various intrinsic and extrinsic factors influence the shelf-life of PMF. Intrinsic factors include the composition of the grain, particularly its lipid content and enzymatic activity, which contribute to rancidity and flavor degradation. Extrinsic factors, such as temperature, humidity, and storage duration, influence microbial contamination and oxidative changes. The susceptibility of PMF to microbial and enzymatic spoilage necessitates careful consideration of these factors to ensure its stability and nutritional quality.

2.4.1. Varieties

The genetic makeup of pearl millet significantly affects its susceptibility to rancidity and deterioration. Different varieties and genotypes exhibit variations in lipid composition, enzymatic activity, and antioxidant capacity, all contributing to differences in shelf-life. Goyal and Chugh (2017) analyzed rancidity parameters, including peroxide value and fat acidity, in various pearl millet hybrids. Their study encompassed seven hybrids (HHB 67 Imp, HHB 94, HHB 146, HHB 197, HHB 223, HHB 226, and HHB 234), seven CMS lines (ICMA 89111, ICMA 95222, ICMA 97111, ICMA 94555, ICMA 843-22, HMS 7A, and ICMA 94222), five inbreds (G-73-107, HTP 94/54, HBL 11, H77/833-2-202, and 78/711), and two white composites (WHC 901-445 and HMP 802). They milled the flour using a cyclone grinder mill and observed that HHB 197 exhibited fewer rancid characteristics compared to others, with fat acidity ranging from 11 to 75 mg KOH/100 g dry mass in fresh flour and 180–330 mg KOH/100 g dry mass in stored flour after 30 days. Additionally, the POD value decreased from 378 to 288 U over 30 days, indicating oxidative changes during storage. These findings highlight that the choice of variety plays a crucial role in determining the shelf stability of PMF, with some genotypes being more resistant to rancidity than others

2.4.2. Storage conditions

Storage conditions, including temperature, humidity, and moisture content, directly impact the quality and longevity of PMF. Joshi and Rao (2024b) studied the effect of different moisture conditions (8%–20% wb), storage temperature (15–45◦C), and humidity (49%–91%) on the quality of pearl millet grains. They advised that the grains (>14% moisture) should be dried to safe levels within 3 weeks at 25 ℃ to maintain quality. When the storage temperature was below 20 ℃ , the seed viability was retained till 15 weeks. These findings underscore the importance of controlled storage conditions in maintaining PMF's freshness and functional properties.

2.4.3. Microbial spoilage

PMF is frequently contaminated and exhibits poor microbiological quality. Pathogenic species in market-sold PMF include Staphylococcus aureus, Escherichia coli, and Bacillus cereus. The microflora in PMF, including fungi (yeasts and molds), aerobic mesophiles, and enterobacteria, originate from poor hygiene and post-harvest practices, leading to flour contamination (Christelle et al. 2021). Pearl millet can be susceptible to mycotoxin and microbial contamination, posing a risk to food safety. Implementing the proper processing parameters and storage will help to reduce the microbial load (Wang et al. 2024). Bacillus subtilis is the primary microorganism in un-malted grain and flour (Badau 2006). On the other hand, S. aureus dominates in PMF. The prevalence of S. aureus in high quantities of food is concerning due to its virulence and safety implications. The minimum cell counts of S. aureus needed to produce the minimum enterotoxin level causing gastroenteritis in humans varies depending on substrates and specific enterotoxins, ranging from 6.7 to 10.7 log CFU/g. The presence of aerobic mesophilic microflora at 5-log CFU/g, fungal flora at 3-log CFU/g, Enterobacteria at 1-log CFU/g, fecal coliforms at 2-log CFU/g, E. coli at 1-log CFU/g, S. aureus at 2-log CFU/g, and B. cereus at 5-log CFU/g has been reported in PMF

(Christelle et al. 2021). In blanched millet flour, the primary organism genera are Micrococcus luteus and Lactobacillus acidophilus (Akinola et al. 2017). Contaminated flour will lead to food intoxication and food poisoning. Thus, effective treatment is essential for maintaining the quality and safety of PMF (Paul et al. 2020).

2.4.4. Enzymatic spoilage

Pearl millet spoilage happens mostly due to enzymatic reactions. The spoilage enzyme activity in this grain varies with the genotypes (Bhargavi et al. 2024). Table 2.6 presents the enzyme activity levels in different varieties of pearl millet, highlighting variations in lipase, POD, LOX, and PPO activity. HHB223 and ICMA95222 exhibit the highest POD activity (595 and 515 U/g, respectively), whereas WHC 901-445 has the highest LOX activity (615 U/g). The lipase activity varies significantly among varieties, with PC443 (47.1 U/g) and Dhanshakti (67 U/g) showing elevated levels compared to others. Lipases are primarily found in the pericarp of the grain (Chitranashi et al. 2024), whereas LOX enzymes are mainly located in the cytoplasm of the grain cells, including the periplasm and inner and outer membranes (Sharma and Chugh 2017) (Figure 2.1). Milling accelerates enzymatic spoilage by removing the outer layers of the grain, where enzymes are concentrated, and by exposing these enzymes to external environmental conditions. The increased surface area enhances their interaction with lipids and other substrates. In milled flour, enzyme activity is intensified due to favorable environmental factors—such as moderate temperatures (30–40 °C), relative humidity above 65%, and the naturally occurring pH of PMF (6.0–6.8)—which collectively promote optimal enzymatic function. Additionally, milling disrupts cellular structures, increasing substrate accessibility and further accelerating enzymatic reactions, thereby making the flour more susceptible to spoilage. Because PMF contains relatively high lipid content (3%–8%), the lipase enzyme plays a key role in breaking down lipids and producing FFA.

Lipase diacylglycerols, monoacylglycerols, glycerol, and FFAs. These FFAs serve as substrates for LOX, which catalyzes the oxidation of polyunsaturated fatty acids, resulting in the formation of lipid hydroperoxide as a primary oxidative product (Goswami et al. 2020; Narayanan and Ravikumar 2017). LOX creates highly reactive molecules that affect the organoleptic characteristics and color of the flour (Figure 2.3). Pearl millet also contains oxidative enzymes such as POD and PPO, which play crucial roles in the deterioration of PMF quality. These enzymes contribute to two rancidity mechanisms: enzymatic and oxidative rancidity, leading to off-flavor development in PMF. Enzymatic rancidity occurs when enzymes like lipases and oxidative enzymes (POD and PPO) catalyze the breakdown of lipids. POD and PPO react with lipid hydroperoxides to produce reactive oxygen species (ROS), which further degrade FFA, accelerating lipid oxidation and rancidity (Goswami et al. 2023). Additionally, PPO catalyzes the oxidation of o-diphenols to o-quinones, which polymerize into pigmented polyphenols, leading to enzymatic browning and discoloration of the flour (Goswami et al. 2024). Oxidative rancidity is a non-enzymatic process that involves the auto-oxidation of unsaturated lipids in PMF. Lipid hydroperoxides decompose into secondary oxidation products such as aldehydes and ketones, which have strong off-flavors, ultimately contributing to flour rancidity during storage. This oxidation occurs spontaneously or is triggered by ROS generated in enzymatic reactions.

Table 2.6: Enzyme activity reported in different varieties of pearl millet.

Variety	Enzyme activity (U/g)				Reference
	Lipase	POD	LOX	PPO	
HHB67 imp		450	600	5.8	Goyal & Chugh (2017)
HHB94		490	610	6.4	
HHB146		485	605	5.9	
HHB197		390	500	5.9	
HHB223		595	585	6.6	
HHB226		400	510	5.5	
HHB234		485	610	5.9	
ICMA89111		295	405	6	
ICMA97111		350	400	4.6	
ICMA843-22		220	495	5	
ICMA 94222		295	505	4.9	
ICMA95222		515	685	6.6	
ICMA94555		600	605	5.2	
HMS7A		405	600	6	
G-73-107		405	500	5.3	
HBL 11		350	500	5.3	
HTP 94/54		390	505	5.05	
H77/833-2-202		350	515	5.8	
78/711		280	510	6.1	
WHC 901-445		500	615	6.4	
HMP802		595	515	5.9	
MPMH 21	1.8				Padmaja et al. (2023)
Dhanashakti	1.1				
HHB67 imp	2.4				
PC443	47.1*				Goswami et al. (2024)
HHB67 imp	20.9*				

GHB732	20.0*				
HHB226	21.9*				
86M01	43.6*				
GHB744	23.4*				
Dhanshakti	67*	110*	199*	0.09*	Vinutha et al. (2022)
SS8484	475*		0.82*		Vinay et al. (2025)

Note: The cell with no data means that the corresponding information is not available in the literature.

Abbreviations: LOX, lipoxygenase; POD, peroxidase; PPO, polyphenol oxidase.

arefers to the unit in (mMol substrate)·min−1·(mg/protein).

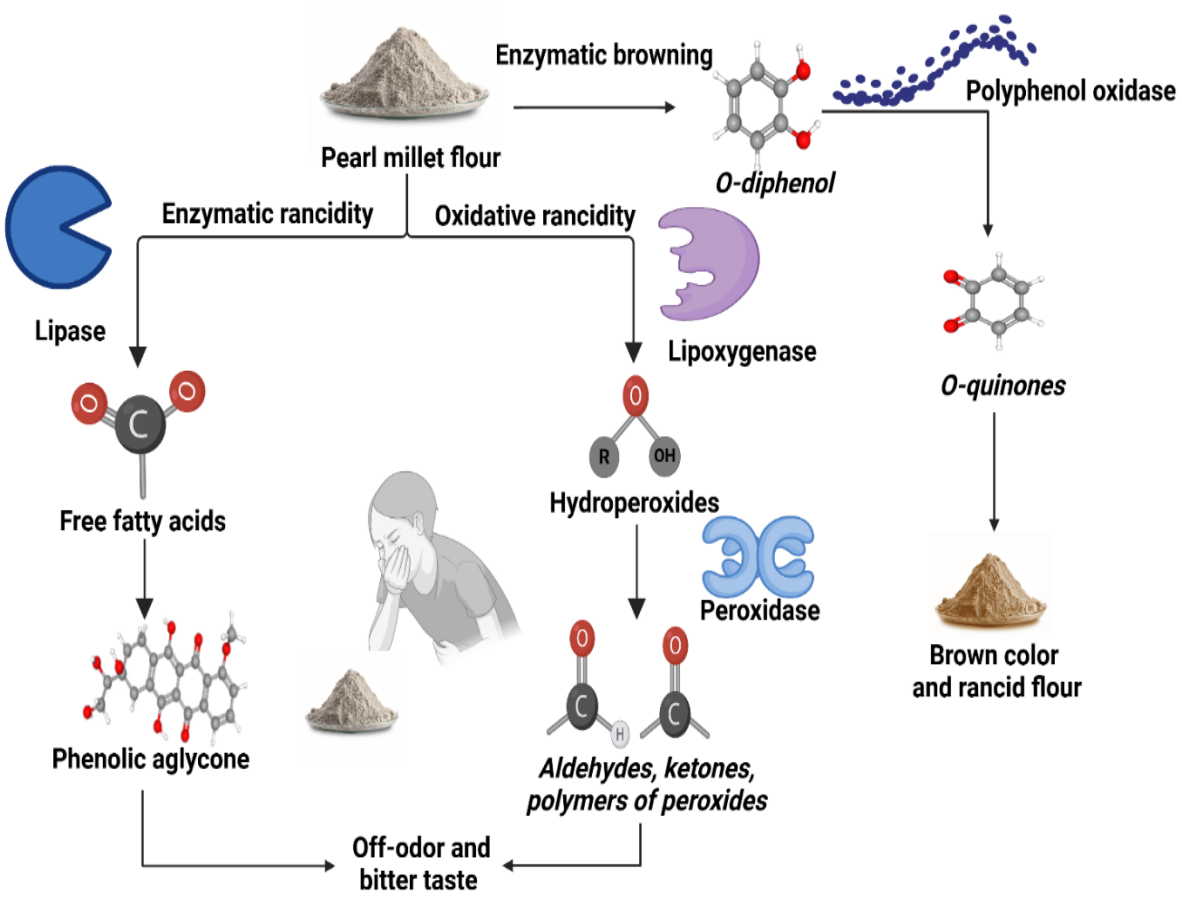

Figure 2.3: Schematic showing various types of enzymatic spoilage occur in pearl millet flour

2.5. Impact of Processing Methods on Nutrients and Anti-nutrients in Pearl Millet Flour

Various processing methods impact both the nutrient composition and anti-nutritional factors of PMF (Table 2.7). Hydration is a crucial step in grain processing, such as in malting, germination, and fermentation, as it facilitates enzyme activation, metabolic processes, and the movement of water and nutrients within the grain (Miano et al., 2018). The decortication step also reduced the antinutrients in PMF by removing the outer layer of the grain, which contains a high concentration of the antinutrient compounds. Traditional thermal techniques such as roasting, steaming, blanching, hydrothermal methods, acid treatment, and dry heat treatment have been used historically, while non-thermal techniques like cold plasma and ultrasound have also been utilized recently. The extent of nutrient retention and anti-nutrient reduction varies across methods, depending on processing conditions.

2.5.1. Fermentation

Fermentation and malting have been traditional methods used to create indigenous fermented and malted products, which have improved nutritional, health, and sensory qualities. Fermented grains, enriched with bioactive components like γ-aminobutyric acid and natural phenolics, exhibit diverse antioxidants and anti-cancer effects. Furthermore, fermentation has been shown to increase antioxidant effects, including free radical-scavenging, reducing, and metal-chelating abilities, by boosting antioxidant phenolics and peptides (Gan et al., 2017). The metabolic activities of microorganisms and enzymes during fermentation and malting processes may contribute to the observed decrease in the anti-nutrient content (Akinola et al., 2017). Chinenye et al. (2017) explored the impact of fermentation, with and without a starter culture (*Lactobacillus plantarum*), on the nutrient properties of the Sosart 1 variety pearl millet stored at 37 °C for 4 days. The optimal time for enhancing nutrient properties was 4 days at 37 °C. They observed a decrease in pH and

an increase in titratable acidity in both naturally fermented and cultured-fermented samples due to the dominance of lactic acid bacteria, which degrade carbohydrates and cause acidification. The reduction in anti-nutrients post-fermentation may be attributed to leaching during soaking. Bhati et al. (2016) studied the impact of malting on the PMF. They reported a significant reduction in polyphenol content during malting, from 675.33 mg/100 g to 303.2 mg/100 g. Choudhary et al. (2023) concluded that fermentation (72 h) was the optimal method for enhancing material properties compared to steaming (30 min), blanching (98 °C, 30 s), and ultrasound (66% amplitude, 26 min). They noted significant reductions in tannin and phytic acid contents by 57% and 84%, respectively. The reduction in fat content during natural fermentation was attributed to increased lipolytic enzyme activity, which breaks down fat constituents into glycerol and fatty acids through hydrolysis. Fermentation increased protein, moisture content, total phenol, and total flavonoid content by 14.7%, 6.8%, 2.5%, and 2.9%, respectively. The increase in protein content during fermentation is partly due to the breakdown of carbohydrates by microorganisms, which release nitrogen resources for protein synthesis. Moreover, reducing carbohydrates redistributes the proportions (on a whole mass basis) of other components, such as protein and fat, potentially leading to higher protein concentration. Additionally, microbial enzymatic activity during fermentation helps break down seed components, freeing nitrogen for protein production (Choudhary et al., 2023). The higher phenolic content during the fermentation of seeds might be due to the enzymatic breakdown of cell walls and other plant components, which can release bound phenolic compounds. Additionally, microbial activity during fermentation can lead to the production of phenolic compounds as secondary metabolites.

2.5.2. Decortication

In millet processing, decortication is a crucial step involving removing the bran and hard cellulosic outer layer stuck to the grain's surface. The decortication process greatly impacts the biochemical and nutritional characteristics of millet grains because of the irregular distribution of nutrients in the grain. Decortication, aimed at removing antinutrients from the cell wall, significantly influences grain nutrient properties (Joshi & Rao, 2024a). Goswami et al. (2024) explored the impact of decortication levels on the nutritional properties of eight diverse pearl millet varieties. A low level (1-3%) of decortication resulted in reductions of 5.9-13.4%, 15%, and 7.1-25% in antioxidant potential, total phenolic content, and comprehensive peroxide value, respectively. Conversely, a high level of decortication (6.1-10%) led to significant losses in zinc (1.9-17.7% loss), iron (4.5-81% loss), and antioxidant potential (5.9-38.9% loss) of the grains.

2.5.3. Thermal treatments

Thermal treatments, including heating, blanching, and roasting, have been extensively researched to determine their impact on the nutrients and anti-nutrients present in PMF. These methods apply heat to the grain, substantially altering the chemical structure of nutrients and anti-nutrients. Obadina et al. (2016) found that grains treated at 180 °C for 10 min showed a higher iron availability. Increasing roasting temperature notably reduced the phenolic content in PMF. The protein content of the control sample surpassed that of other roasted milled flour samples, possibly due to structural changes in endogenous proteins during roasting**,** leading to amino acid alterations due to heat. Ash content in samples ranged from 1.8 to 2.2%, which increased with temperature. Heat-sensitive lysine content decreased post-treatment. Roasting also reduced methionine and phenolics in PMF. However, roasting increased ash and carbohydrate content without affecting fat, threonine, and glycine levels.

Different thermal treatments impart various positive characteristics to the grains by increasing the available minerals and nutrient properties. Bhati et al. (2016) studied the impact of different treatments on pearl millet grains, such as the dry heat treatment (30-120 min) and blanching treatment (100 °C for 30-90 s). The maximum iron content, 3.58 mg/100 g, was found in the dry heat treatment lasting 120 min. The minimum reduction of polyphenols, 658.6 mg/100 g, occurred during the 30-min dry heat treatment, resulting in the lowest FFA content at 33.0 mg/100 g. The highest *in vitro* iron concentration was found after 120 min of dry heat treatment, followed by 90 s blanching treatment. However, grains subjected to 120 min dry heat treatment exhibited a darker color and a cooked flavor. Thus, the authors recommended 90 s of blanching treatment for product development due to its high *in vitro* iron content (3.29 mg/100 g), substantial reduction in free fatty acids (20.57 mg/100 g), color improvement, and enhanced sensory attributes like color. Prashanth et al. (2024b) conducted a hydrothermal treatment of pearl millet grain (soaking for 3 h at 55 °C + steaming for 15 min) and observed only 5.9% and 6% reduction in tannins and phytates, respectively.

2.5.4. Nonthermal processing methods

Few non-thermal techniques, such as ultrasound and cold plasma treatment, have been employed so far on PMF. For instance, Sarkar et al. (2023) exposed PMF to cold plasma (20-30 kV for 10-20 min) and analyzed its anti-nutrients. The sample exposed at 30 kV for 20 min showed the highest reduction in anti-nutrient properties. The reduction of tannin and phytate content was 17% and 27%, respectively. The 6.7% reduction in moisture content may be attributed to the dissociation of water molecules under high-energy conditions, leading to the formation of reactive oxygen species (ROS) such as hydroxyl radicals (•OH) and superoxide anions (O_2^-). Moisture content typically includes free and bound water, with the free water being more easily lost. In the

case of cold plasma treatment, water molecules are dissociated by reactive species, such as hydroxyl radicals and ozone. These activated molecules become more mobile, potentially reducing the overall moisture content. Additionally, starch granules, which have a high capacity to absorb and retain water, may have dissociated and taken up more water molecules, further contributing to the observed moisture reduction. The protein content in the samples increased from 10.8% (control) to 12.4% (30 kV/20 min), while fat concentration increased from 5.4% (control) to 7.8% (30 kV/20 min).

The observed increase in protein concentration, despite the reduction in moisture content, is likely a result of the concentration effect—as water is lost, the proportion of remaining solids, including proteins, increases. It is important to note that protein content was measured on a wet basis rather than a dry mass basis, which may partially explain the apparent increase. Cold plasma treatment reduces the water content in the sample, which can make existing protein appear more concentrated. In addition to this physical concentration effect, reactive species generated during cold plasma treatment—such as atomic oxygen (O), hydroxyl radicals (•OH), and ozone (O_3)—can interact with protein structures. These interactions may alter protein conformation, solubility and even induce partial denaturation or aggregation, which could influence how proteins are detected or quantified during analysis. The reported increase in fat content after treatment is more complex. While the author suggests it may result from the breakdown of crude fats into their derivatives, this change raises questions, given the principle of mass conservation. In theory, the total fat mass should remain constant unless there is a loss or transformation. However, cold plasma may disrupt lipid–protein or lipid–starch complexes or break down aggregated lipids into simpler, more extractable forms. This could increase the efficiency of fat extraction by the solvent used in crude fat estimation, leading to a higher measured fat content. Furthermore, cold plasma

can induce microstructural changes, such as the dissociation of starch granules or matrix loosening, which may facilitate the release of bound lipids or other components. Thus, both physical redistribution within the matrix and enhanced extractability may explain the observed variation in protein and fat content after treatment. Furthermore, carbohydrate content decreased from 66.9% to 65.6%, with a pH reduction from 7.8 to 6.9. They also observed a decrease of 15.5% in total flavonoids and 15% in phenolic compounds. Cold plasma treatment significantly improved the L^* value, with the highest value observed at 30 kV for 20 min (75.84) compared to the control (74.73). The a^* and b^* values of cold plasma-treated PMF decreased with longer treatment durations, while the L^* value increased. This is likely due to ROS oxidizing the conjugated double bonds in carotenoids, causing pigment degradation. As the carotenoid pigments break down, the flour loses its vibrant pigmentation, resulting in a paler, more yellow appearance. Lokeswari et al. (2021) exposed PMF to cold plasma treatment at 40 and 45 kV for 5, 10, and 15 min. Based on various attributes, including enhanced swelling capacity, water absorption, and pasting properties of PMF, they recommended the optimal condition as 45 kV for 15 min. The fat content increased from 3.4% to 4.7%, while crude fiber and protein content decreased from 2.2% to 1.3% and 10.0% to 8.9%, respectively. There are contradictory reports on changes in protein content after cold plasma treatment. The variations in protein content can be attributed to differences in experimental conditions, such as treatment duration, plasma intensity, and the type of plasma applied. These factors influence how reactive species interact with the grain's cellular structure, potentially leading to either the breakdown of protein or minimal changes, depending on the severity of the treatment. The color intensity of the grains increased with treatment, although the change in chroma decreased with longer treatment times. These color changes are primarily attributed to the oxidation of pigments by ROS, driven by high-energy reactive species during treatment. Starch

hydrolysis occurs, breaking down starch into sugar fragments and acids. Together, these biochemical transformations contribute to the color changes and highlight the complexity of cold plasma treatment effects on grains.

Charu et al. (2024) evaluated the impact of cold plasma treatment at 2 kV for 5 min on pearl millet grains. They discovered considerable reductions in tannin and phytic acid concentrations of 18% and 57%, respectively. In contrast, the fat content increased by 43%, while the protein content was almost similar (from 12.6% to 12.5%). Notably, vitamin B_{12}, B_3, and B_9 levels increased by 9.5%, 0.6%, and 40.4%, respectively. The study also noted a reduction in total flavonoids and phenolic content, 10.6% and 2.1%, respectively. Meanwhile, phosphorus and iron content increased, while magnesium and calcium content decreased. The reduction in antioxidant activity after cold plasma treatment is linked to hydroxylated and quinone compounds forming from O_3 interaction with phenolics. This interaction, alongside oxidative degradation by ROS generated during treatment, reduced total flavonoid content. The study also proposed that ROS may disrupt glycosidic bonds, thereby reducing tannin and phytic acid levels in the grains. These findings highlight the intricate biochemical changes induced by cold plasma treatment on grain composition and antioxidant properties.

Table 2.7: Effect of various thermal and nonthermal processing methods on nutrients and anti-nutrients in pearl millet flour.

Variety	Treatment applied	Optimum condition	Nutrient properties	Changes	Other observation	References
PC 433, HHB 67 imp, GHB 732, HHB 226, Dhanshakti, MPMH 17, 86M01, and GHB 74	Decortication: low, middle, and high with 1–3%, 3.1-6, and 6.1-10% weight loss, respectively.	Low level of decortication	Antioxidants Total phenolics Iron Zinc Peroxide value	100 to 86.6% 100 to 85.0% 100 to 94.1% 100 to 96.7% 7 to 25%	More loss of zinc, iron, and antioxidant potential occurs in the high level of decortication.	Goswami et al. (2024)
NM	Cold plasma (20-30 kV for 10-20 min)	30 kV for 20 min	Moisture content Protein Fat Carbohydrates Tannin Phytate pH	10.7 to 10.0% 10.7% to 12.4% 5.5% to 7.8% 66.9% to 65.7% 0.98 to 0.81% 9.25 to 6.71% 7.83 – 6.97	A reduction in total flavonoids and phenolic compounds is observed.	Sarkar et al. (2023)
NM	Cold plasma (40-45 kV for 5-15 min)	45 kV for 15 min	Fat Crude fiber Protein Moisture	3.4 to 4.7% 2.2 to 1.3% 10.0 to 8.9% 11.9 to 10.2%	Color intensity increased with treatment.	Lokeswari et al. (2021)

Sosart 1	Fermentation with *Lactobacillus plantarum*	4 days at 37 °C	pH Titratable acidity Protein Phytate Phytic acid Moisture Ash Lipid	3.64 to 3.48 0.09 to 0.82% 20.5% 0.09% 1.40% 7.58% 0.34% 0.64%		Chinenye et al. (2017)
NM	Acid treatment (2-24 h)	Acid treatment (24 h)	Iron Free fatty acids	119.3 to 2.39 mg/kg 445.6 to 116 mg/kg	It improved the color of the grains.	Bhati et al. (2016)
NM	Dry heat (30-120 min)	Dry heat (120 min)	Iron Free fatty acids	119.3 to 35.8 mg/kg 445.6 to 330.2 mg/kg	It led to a darker color.	Bhati et al. (2016)
Dhanashakti	Cold plasma at 2 kV for 5 min	-	Tannin Phytic acid Fat Moisture Vit B12 Vit B3 Vit B9 Protein Carbohydrates Ash Total flavonoids Total phenolics Phosphorus Calcium Iron Magnesium	100 to 82% 100 to 43% 100 to 143% 5.6 to 4.1% 2.41 to 2.64 mg/kg 9.39 to 9.45 mg/kg 7.95 to13.34 mg/kg 12.6 to 12.5% 77.1 to 77.0% 1.78 to 1.79% 3030 to 2710 mg/kg 840 to 820 mg/kg 4082 to 3921 mg/kg 188 to171 mg/kg 48 to 49 mg/kg 1247 to 1230 mg/kg	Decrease in antioxidant activity.	Charu et al. (2024)

Dhanashakti	Fermentation (72 h), steaming (30 min), blanching (98 °C/30 s) ultrasound (66% amplitude, 26 min)	Fermentation (72 h)	Tannin Phytic acid Protein Moisture Fat Ash Carbohydrates Energy Total phenolics Total flavonoids	2360 to 1010 mg/kg 100 to 16% 12.5 to14.7% 5.6 to 6.8% 2.7 to 1.4% 1.7 to 1.5% 77.1 to 75.3% 384 to 373 kcal/g 840 to 860 mg/kg 3030 to 3120 mg/kg	Ultrasound enhanced color and antioxidant activity.	Choudhary (2023)
L.R.Br. variety	Roasting (120-180 °C for 3-15 min)	Roasted at 180°C for 10 min	Protein Total ash Crude fiber Moisture Fat Lysine Methionine Phenol content	8.3 to 7.4 % 2.2 to 1.7% 1.0 to 0.5%, 12.3 to 11.0% 7.0 to 6.9% 2.6 to 1.0 g/kg 0.6 to 0.1 g/kg 1690 to 900 mg/kg	Iron content increased at 180 °C.	Obadina et al. (2016)
HHB 67	Hydrothermal treatment (35-55 °C; 2-4 h; steaming time 5-15 min)	Soaking for 4 h at 55 °C and steaming for 15 min	Tannin Phytate	5.9% 6%	Steaming for 15 min decreased the degree of gelatinization.	Prashanth et al. (2024b)

NM, not mentioned in

2.6. Impact of Various Processing Methods on Functional Properties of Pearl Millet Flour

The functional properties of the flour are important to improve the textural properties and acceptance of the final product (Table 2.8). Several types of research have been conducted on WAC, OAC, emulsifying capacity, foaming capacity, swelling capacity, and water solubility index of PMF. Understanding these functional properties is crucial for effective product development. The changes in functional properties of PMF after any treatment have been summarized below.

2.6.1. Fermentation and malting

Germination, fermentation, and malting processes are employed to enhance not only the functional properties but also the digestibility of the material, making them essential steps in improving overall product quality (Akinola et al., 2017). Seed sprouting enhances their nutritional properties and increases essential nutrient bioavailability. It makes the flour suitable for various food applications by enhancing its digestibility and reducing the antinutritional factors (Lemmens et al.,2019). Adebiyi et al. (2017) researched the impact of fermentation (1:4 ratio of water and grains at 28 °C for 3 days) and malting (soaking in water for 24 h followed by germination) on pearl millet grains. Both treatments improved the functional properties, with malted flour exhibiting higher WAC (2.26 g/g), OAC (1.29 g/g), and swelling capacity (23.5 mL) compared to fermented flour. Malted flour (bulk density 0.69 g/cm^3) also demonstrated lower bulk density compared to both the control (0.78 g/cm^3) and fermented flour (0.76 g/cm^3). The structure of non-fermented flour showed smaller, irregular, and compact granules, while malting and fermentation processes induced changes, resulting in a more regular granular structure in their respective samples. This indicates that fermented and malted flours have a greater affinity for water due to changes in starch polymers, leading to higher WAC. Similar trends were observed for OAC, where physical oil entrapment and binding to the protein's polar chain play significant roles. Enzymatic actions during these processes contribute to starch dextrinization, breaking it into smaller subunits. This results

in less bulky flours with a higher nutrient density. Higher WAC, OAC, foaming, and emulsifying capacity will improve the final product's textural aspects. OAC is crucial in various applications such as baking, frying, and confectionary foods. It can enhance texture, flavor retention, and shelf stability of food products, making the flour more versatile for different recipes.

2.6.2. Thermal treatment

Thermal treatments involve subjecting the grains to controlled heat, leading to biochemical and structural changes that impact the various functional attributes of the flour. Roasting is a common processing technique that can significantly impact the functional properties of the flour. Obadina et al. (2016) roasted pearl millet grains at 120-180 °C for 3 to 15 min. Roasting at 180 °C/10 min increased the water solubility index (5.1 to 7.7%) and OAC (0.3 to 0.6%). Higher WAC promotes bulkiness and consistency, making PMF susitable for baking. The change in water solubility may arise from altered nutrient bioavailability of proteins from roasted flour. Prashanth et al. (2024a) employed hydrothermal treatment of pearl millet grain involving soaking for 3 h at 55 °C and steaming for 15 min. This led to a WAC of 3.33 g/g, OAC of 2.1 g/g, water solubility of 6.16%, and emulsion capacity of 72.7%, with a noted decrease in water absorption and solubility as soaking time increased.

2.6.3. Nonthermal treatment

Nonthermal treatments for pearl millet flour or grain have not been extensively explored. Few studies reported that the cold plasma treatment improved the viscosity and pasting profile of the pearl millet grain. For instance, Sarkar et al. (2023) exposed the pearl millet grains to cold plasma treatment at 30 kV for 20 min. They reported enhancement in water absorption (22%), oil absorption (18%), emulsifying capacity (5%), and foaming capacity (20%). The crystallinity of the grains decreased after the treatments. It was showing a shear thinning behavior, thus indicating

an elastic response. This suggests that cold plasma treatment could also enhance the process by contributing to enzymatic stability and improving functional properties. Increased voltage correlated with enhanced gelatinization of starch, resulting in higher viscosity, consistency, and lower flow behavior in treated samples. The presence of more hydrophilic sites like proteins, carbohydrates, and specific residues of polar amino acids in the cold plasma-treated samples might contribute to these changes. Similarly, Lokeswari et al. (2021) found that cold plasma treatment improved the swelling capacity, solubility, and water absorption capacity of pearl millet grains by 0.2-0.3%, 183-212%, and 1.1-1.5%, respectively. Swelling capacity reflects the interactions among starch chains in different forms, such as crystalline and amorphous. This further supports the idea that cold plasma treatment can improve the functional properties and help reduce spoilage by enzyme activity, aiding in enzymatic stability. Changes in amylose-to-amylopectin ratios concerning molecular weight, branch length, and branching degree play a role in swelling power. The increased WAC in cold plasma-treated samples may be attributed to elevated levels of hydrophilic components like carbohydrates, proteins, and specific polar amino acid residues, enhancing their affinity for water molecules. These findings collectively emphasize the potential of cold plasma treatment in improving the functional properties of pearl millet grains. A few nonthermal methods, such as high-pressure processing (HPP) and ozone treatment, have been applied not directly to PMF but to starch extracted from it. Thakur et al. (2023) investigated the effect of ozonation (0.06–0.1 L/min for 60 min) on isolated pearl millet starch. Swelling power increases with temperature but decreases with higher ozone exposure. Ozone treatment reduced in vitro starch digestibility from 44.59 to 29.20 g/100 g maltose. Treated starch exhibited higher peak and final viscosity but lower breakdown and setback viscosity. While the crystalline pattern remained unchanged, relative crystallinity decreased. Additionally, HPP increased the water-

holding capacity of PMF starch from 0.66% to 2.19%, indicating improved hydration properties (Mirzababaee et al., 2022). The reduced retrogradation tendency and increased elasticity of starch under HPP further suggest its potential to enhance textural attributes in processed foods.

Table 2.8: Effect of various thermal and nonthermal processing methods on functional properties of pearl millet

Pearl millet	Treatment applied	Optimum condition	Functional properties	Changes	Other observation	References
NM	Cold plasma (20-30 kV for 10-20 min)	30 kV for 2 min	Water absorption Oil absorption Emulsification Foaming Crystallinity	1.32 to 1.62 g/g 1.11 to 1.31 g/g 86.5 to 91.2% 10.6 to 12.8% 23.4 to 19.3%	The treated sample showed a shear-thinning behavior.	Sarkar et al. (2023)
NM	Cold plasma (40-45 kV for 5-15 min)	45 kV for 15 min	Swelling capacity Solubility Water absorption	0.21 to 0.32% 183 to 212 % 1.11 to 1.51%		Lokeswari et al. (2021)
NM	Malting	Malting in water for 24 h + germination	Water absorption Oil absorption Swelling Bulk density	1.32 to 2.26 g/g 1.20 to 1.29 g/g 12.5 to 23.5 mL 780 to 690 kg/m^3	Sample had the regular structural network after malting	Adebiyi et al. (2016)
NM	Fermentation	Fermentation of grains in 4-times water for 3 days at 28°C	Water absorption Oil absorption Swelling Bulk density	1.32 to 1.66 g/g 1.20 to 1.24 g/g 12.5 to 13.5 mL 780 to 760 kg/m^3	Fermented grains showed a smoother structural network.	Adebiyi et al. (2016)

L.R.Br.	Roasting (120-180 °C for 3-15 min)	Roasting at 180 °C for 10 min	Water solubility Oil absorption	5.12 to 7.76% 0.33 to 0.68%		Obadina et al. (2016)
HHB 67	Hydrothermal treatment (35-55 °C; 2-4 h; steaming time 5-15 min)	Soaking for 3 h at 55 °C and steaming for 15 min	Water absorption Oil absorption Water solubility Emulsion capacity	3.33 g/g 2.1 g/g 6.16% 72.7%	Water absorption and solubility decreased with soaking time.	Prashanth et al. (2024a)

NM, not mentioned in the study.

2.7. Effect of Various Processing Methods on Microbial Safety of Pearl Millet Flour

Ensuring the microbial safety of PMF is crucial for preventing food-borne illness and food intoxication. Various methods such as fermentation, thermal (blanching, steaming, and roasting), nonthermal (cold plasma and ultrasound), and combined thermal and nonthermal (dry heat+ irradiation) treatments have been employed to reduce the microbial load in the pearl millet or PMF (Table 2.9). The effectiveness of each treatment is measured in terms of microbial reduction (log CFU/g), targeting different microorganisms such as aerobic mesophiles, yeast, coliforms, *Enterococcus* spp, and fungi.

2.7.1. Fermentation

Fermentation of pearl millet at 25 °C for 72 h, followed by drying at 50 °C, significantly reduced the microbial load (Choudhary et al., 2023). The highest log reduction of the aerobic mesophiles was achieved through fermentation, decreasing from 7.61 to 2.36 log CFU/g, resulting in a 5.25 log reduction. Fermentation effectively lowers the microbial population through pH reduction, antimicrobial compound production, and the generation of ethanol and CO_2. In particular, lactic acid fermentation is crucial in inhibiting the growth of various pathogenic bacteria, viability, and toxin production. An effective fermentation process can achieve a pH below 4, creating an acidic environment that suppresses bacterial pathogens, leading to their gradual decline. Additionally, coliform and *Enterococcus* counts decreased by 4.17 and 3.0 log CFU/g, respectively. Fermentation also had beneficial effects on nutritional properties, increasing phenolic content and reducing phytates by 84%.

2.7.2. Thermal treatments

Blanching, steaming, and roasting also demonstrated microbial inactivation in PMF. Blanching (hot water at 98 °C for 30 s) led to a reduction of aerobic mesophiles by 4.45 log CFU/g, yeast by

4.14 log CFU/g, and coliforms by 4.47 log CFU/g while also reducing phytates by 72% (Choudhary et al., 2023). Open steaming (100 °C for 20 min) was slightly more effective than blanching, achieving a coliform reduction of 5.38 log CFU/g and aerobic mesophiles reduction of 4.71 log CFU/g. Roasting at medium flame (~180 °C) for 10 min significantly reduced aerobic mesophiles by 4.69 log CFU/g, with microbial counts dropping below detectable limits at a 10^{-5} dilution (Narayanan & Ravikumar, 2017). However, heat treatment using a rotary dryer at 150-170 °C for 1.5 min showed only minimal fungal reduction (0.28 log CFU/g) (Dikkala et al., 2018). Among thermal treatments, steaming was more effective than blanching, while roasting achieved near-complete microbial elimination at higher temperatures.

2.7.3. Nonthermal treatments

There are very limited studies on the nonthermal microbial safety of PMF. Nonthermal processing, such as cold plasma and ultrasound, was effective in microbial inactivation. For instance, Charu et al. (2024) explored the effect of cold plasma treatment on microbial reduction of the Dhanashakti pearl millet variety. They achieved a log reduction from 7.61 log CFU/g to 1.78 log CFU/g (5.83 log reduction) and from 6.43 log CFU/g to 1.95 log CFU/g (4.48 log reduction) in the aerobic mesophilic count and total yeast count, respectively, after a cold plasma treatment at 2 kV/5 min. However, the most significant decrease was observed in the counts of *Enterococcus* spp (3.6 log) and *E. coli* (3.47 log) at 2 kV/5 min. Choudhary et al. (2023) explored ultrasound treatment (20 kHz, 66% amplitude, 26 min) on the Dhanashakti pearl millet variety. Ultrasound treatment resulted in aerobic mesophilic count reduction of 4.83 log CFU/g, yeast by 4.61 log CFU/g, and coliforms by 4.53 log CFU/g. Additionally, ultrasound enhanced antioxidant activity and vitamin content while decreasing phytates by 70%.

2.7.4. Combined treatment

A combination of thermal and nonthermal treatment showed potential in microbial inactivation in pearl millet. For instance, Dikkala et al. (2018) employed heat treatment (150-170 °C for 1.5 min) combined with irradiation (2.5 kGy) on pearl millet. It showed improved fungal inactivation, achieving a 1.25 log reduction, whereas a lower irradiation dose (1 kGy) resulted in only a 0.5 log reduction.

Different processing methods impact microbial reduction in distinct ways. Roasting effectively reduces microbial load but can degrade heat-sensitive vitamins. Fermentation inhibits pathogenic microbes while promoting beneficial activity; however, its effectiveness varies depending on microbial species and requires controlled conditions for safety and consistency. Lactic acid fermentation, for instance, is particularly effective against enteric pathogens due to pH reduction and antimicrobial compound production, but its efficacy against heat-resistant spores is limited. Blanching inactivates both enzymes and microbes but results in the gradual loss of water-soluble vitamins. Cold plasma treatment is highly effective in reducing surface microbes, but moisture levels during storage must be carefully managed to prevent recontamination. While fermentation offers a substantial microbial reduction, its effectiveness should be compared with other methods based on the target microorganism and storage considerations. However, very few studies have explored microbial inactivation in PMF. While fermentation offers substantial microbial reduction, its effectiveness must be evaluated alongside other methods, considering the target microorganisms and storage conditions. However, limited research has specifically addressed microbial inactivation in PMF, especially using combined or novel treatments. Therefore, additional studies are needed to explore optimized, synergistic processing strategies that ensure microbial safety without compromising nutritional or functional quality.

Table 2.9: Effect of various thermal and nonthermal processing methods on microbial safety of pearl millet flour.

Treatment	Conditions	Variety	Microorganism	Microbial reduction (log CFU/g)	Other observations	Reference
Fermentation	Fermentation at 25 °C for 72 h + drying at 50 °C for 4 h	Dhanshakthi	AM Yeast Coliform *Enterococcus*	AM: 5.25 Yeast: 5.09 Coliform: 4.17 *Enterococcus*: 3.0	- Phytates reduced by 84%. - Phenolics increased.	Choudhary et al. (2023)
Blanching	Hot water at 98 °C for 30 s + drying at 50 °C for 1 h	Dhanshakthi	AM Yeast Coliform *Enterococcus*	AM: 4.45 Yeast: 4.14 Coliform: 4.47 *Enterococcus*: 3.09	- Phytates reduced by 72%.	Choudhary et al. (2023)
Steam treatment	Open steaming (100 °C) for 20 min + drying at 60 °C for 1 h	Dhanshakthi	AM Yeast Coliform *Enterococcus*	AM: 4.71 Yeast: 4.64 Coliform: 5.38 *Enterococcus*: 4.9	- Phytates reduced by 74%.	Choudhary et al. (2023)
Heat treatment	150-170 °C for 1.5 min in a rotary dryer	NM	Fungi	Fungi: 0.28	-No significant difference between dehulled and whole grain decontamination	Dikkala et al. (2018)
Roasting	Medium flame ~180 ºC for 10 min	Dhanshakthi	AM	AM: 4.69	-Post roasting, the microbial count was below the detection limit at 10^{-5} dilution	Narayanan & Ravikumar (2017)
		Proagro	AM	AM: 4.69		
Cold plasma	2 kV, 5 min	Dhanashakti	AM Yeast Coliform *Enterococcus*	AM: 5.82 Yeast: 4.47 Coliform: 3.47 *Enterococcus*: 3.6	-Maximum inactivation was for *Enterococcus* spp. and *E. coli*	Charu et al. (2024)
Ultrasound	20 kHz, 66% amplitude, 26 min + drying at 50 °C for 1 h	Dhanshakthi	AM Yeast Coliform *Enterococcus*	AM: 4.83 Yeast: 4.61 Coliform: 4.53 *Enterococcus*: 4.12	-Antioxidant activity and vitamin content increased. - Phytates decreased by 70%.	Choudhary et al. (2023)

Heat treatment + Irradiation	150-170 °C for 1.5 min in a rotary dryer + 2.5 kGy	NM	Fungi	1.25	- 1 kGy showed a 0.5 log reduction	Dikkala et al. (2018)

AM, Aerobic mesophiles; NM, not mentioned.

2.8. Effect of Various Processing Methods on Enzyme Inactivation in Pearl Millet Flour

Enzyme activity plays a crucial role in the spoilage of PMF, and various treatments can impact enzyme activity to extend its shelf life. The effects of various mechanical (decortication), thermal, and combined thermal processing methods on enzyme inactivation in PMF have been summarized in Table 2.10. The extent of enzyme inactivation varies based on the treatment type, processing conditions, and millet variety.

2.8.1. Decortication

Decortication, a mechanical processing method, showed varied effects on enzyme inactivation depending on the variety. Goswami et al. (2024) reported that while decortication resulted in a 6.1–10% mass loss, it led to lipase inactivation ranging from 45.9% to 71.9%, with LOX inactivation being relatively lower (0.1–6.2%). GHB732 and HHB226 exhibited the highest lipase inactivation (71.9% and 69.6%, respectively), while GHB744 and PC 433 showed lower reductions (45.9% and 47.7%). Despite partial lipase inactivation, a significant decrease in the acid value (50–64.5%) suggests improved oxidative stability. However, the associated loss of phenolic compounds (39.6–45.6%) reduced the antioxidant potential, which may impact the overall functional properties of the flour.

2.8.2. Thermal treatments

Among thermal treatments, roasting at 150 °C for 30 min resulted in a 58% reduction in lipase activity and a 50% decrease in FFA (Padmaja et al., 2023). Prashanth et al. (2024b) reported that the hydrothermal treatment (soaking for 4 h at 55 °C) followed by steaming for 15 min resulted in only 17% reduction in lipase activity in the pearl millet. This reduction cannot significantly impact rancidity, as lipase remains active enough to hydrolyze fats and contribute to lipid degradation. A greater reduction in lipase activity would be necessary to effectively control rancidity and improve

flour stability. However, stronger hydrothermal treatments (soaking at 30 °C for 1 h followed by steaming at 100 °C for 15 min at 15 psi) resulted in significant inactivation of PPO (54.5%) and POD (99.7%) (Vinutha et al., 2022). This suggests hydrothermal treatments can effectively target oxidoreductases, but lipolytic enzymes require more intense treatments for substantial inactivation. Infrared and microwave treatments also demonstrated notable effects on enzyme activity. Infrared processing (480 W/m² for 5 min at 40 °C) resulted in high POD (99.3%) and PPO (95.5%) inactivation but only moderate reductions in lipase (31.6%) and LOX (60.2%). In contrast, microwave treatment (90 s at 300 W) showed a higher lipase inactivation (85.4%) along with a significant decrease in phytates (16.3%) and tannins (87.3%) (Sundaresan et al., 2025). This suggests microwave processing might more effectively target lipolytic enzymes than infrared treatments.

Advanced thermal techniques such as hot air-assisted radio frequency and halogen air fryer treatments provided the highest levels of enzyme inactivation. Hot air-assisted radio frequency processing at 57.5 °C for 15 min inactivated 97% lipase, indicating its potential as an effective pretreatment for enhancing flour stability (Yarrakula et al., 2022). Similarly, halogen air fryer treatment at 150 °C for 4 min resulted in nearly complete inactivation of lipase (98%) and LOX (100%) (Vinay et al., 2025). However, samples processed beyond 120 °C for 4 min became darker, which may affect consumer acceptability.

2.8.3. Combined thermal treatments

Hydrothermal, followed by infrared processing, demonstrated enhanced enzyme inactivation. Soaking at 30 °C for 1 h followed by steaming (100 °C, 15 min at 15 psi) and subsequent infrared exposure (480 W/m² for 5 min at 40 °C) resulted in 47.8% lipase and 84.8% LOX inactivation, with PPO being completely inactivated (100%) (Vinutha et al., 2022). However, these treatments

also reduced peak viscosity by 20%, suggesting potential changes in functional properties that may affect processing and product formulation.

Various processing techniques vary in their effectiveness in enzyme inactivation, with microwave and advanced thermal treatments (hot air-assisted and halogen air frying) showing the highest lipase reduction. The research on nonthermal enzyme inactivation in PMF is scarce. Decortication and hydrothermal treatments alone are insufficient to significantly inactivate lipolytic enzymes, but their combination with other methods, such as infrared processing, enhance enzyme inactivation. Additionally, varietal differences influence enzyme inactivation levels, with certain varieties like GHB732 and HHB226 exhibiting greater lipase reductions under decortication than others. Understanding these processing effects on enzyme activity is crucial for optimizing PMF stability.

Table 2.10: Effect of various mechanical, thermal, and nonthermal processing methods on enzyme activity in pearl millet flour

Variety	Treatment type	Treatment condition	Enzyme inactivation	Other observations	References
PC 433	Decortication	6.1-10% loss	Lipase: 47.7% LOX: 5.1%	-Comprehensive acid value decreased by 50-64.5%	Goswami et al. (2024)
HHB67-Imp	Decortication	6.1-10% loss	Lipase: 67.2% LOX: 4.9%	-Phenolics decreased by 39.6-45.6%	
GHB732	Decortication	6.1-10% loss	Lipase: 71.9% LOX: 6.2%	-Antioxidant potential decreased by 21.9-38.9%	
HHB226	Decortication	6.1-10% loss	Lipase: 69.6% LOX: 2.6%		
Dhanshakti	Decortication	6.1-10% loss	Lipase: 65.3% LOX: 0.2%		
MPMH17	Decortication	6.1-10% loss	Lipase: 58.3% LOX: 3.3%		
86M01	Decortication	6.1-10% loss	Lipase: 53% LOX: 4.2%		
GHB744	Decortication	6.1-10% loss	Lipase: 45.9% LOX: 0.1%		
HHB67	Roasting	150 °C for 30 min	Lipase: 58%	-50% reduction in FFA	Padmaja et al. (2023)
HHB 67	Hydrothermal	Soaking for 4 h at 55 °C + steaming for 10 min	Lipase: 17.1%	-Lightness decreased from 66.6 to 57.6 due to browning	Prashanth et al. (2024b)
NR	Hot air–assisted radio frequency treatment	57.5 ℃, 15 min with 1.13 m/s air velocity, conveyor speed 25 m/h	Lipase: 97%	The heating rate of 5.2 ℃/min at 15% moisture was optimum.	Yarrakula et al. (2022)
NR	Microwave	90 s at 300 W	Lipase: 85.4%	-16.3 and 87.3% decrease in phytate and tannins, respectively.	Sundaresan et al. (2025)
SS8484	Halogen Air Frier Treatment	150 ℃, 4 min	Lipase: 98% LOX: 100%	-The sample becomes darker beyond 120 ℃/4 min	Vinay et al. (2025)

Dhanshakti	Hydrothermal treatment	Soaking (30 ºC/1 h) + thermal treatment (100 ºC, 15 min at 15 psi) + drying at 35 ºC for 2 h	Lipase: 12.8% LOX: 57.2% POD: 99.7% PPO: 54.5%	- Starch digestibility enhanced by 67%.	Vinutha et al. (2022)
	Infrared treatment	Infrared (480 W/m^2 for 5 min at 40 ºC)	Lipase: 31.6% LOX: 60.2% POD: 99.3% PPO: 95.5%	-Peak viscosity decreased by 33%.	
	Hydrothermal + infrared treatments	Soaking (30 ºC/1 h) + thermal treatment (100 ºC, 15 min at 15 psi) + infrared (480 W/m^2 for 5 min at 40 ºC)	Lipase: 47.8% LOX: 84.8% POD: 98.1% PPO: 100%	-Peak viscosity decreased by 20%.	

NR, not reported; LOX, lipoxygenase; POD, peroxidase; PPO, polyphenol oxidase.

2.9. Shelf life of Pearl Millet Flour after Various Treatments

Some studies employed various techniques to enhance the shelf life of PMF. The examples include decortication, thermal treatments (blanching, microwave, roasting, hydrothermal, and dry heating), nonthermal treatment (irradiation), antioxidant treatment, and a combination of thermal or nonthermal processes (Table 2.11). The extent of the rancidity, color change, and odor decided the shelf life of the pearl millet and the flour.

2.9.1. Decortication

Decortication, a process that removes the grain's outer layers, is another method that can significantly alter the nutrient and anti-nutrient composition of PMF by reducing fiber content and enhancing the availability of inner nutrients. Goswami et al. (2024) investigated the effect of different levels of decortication on eight diverse pearl millet genotypes. They subjected the grains to low (1–3% weight loss: LD), medium (3.1–6% weight loss: MD), and high (6.1–10% weight loss: HD) levels of decortication. Milled flour was stored at 25 ºC up to 10 days. There was a reduction in the lipase and LOX activity, peroxide value**,** antioxidant potential, and total phenolic content, respectively, after decortication. The PC 443 variety exhibited lower susceptibility to rancidity, particularly in medium and highly decorticated grains, due to its lower fat content (5.19%), which reduces the availability of lipids for oxidation (Kumari et al., 2018). HD effectively enhanced the shelf life of pearl millet flour. Specifically, LOX activity, comprehensive peroxide value, and malondialdehyde content decreased by 11.2%, 12.5%, and 9.7%, respectively, by the 10th day of storage. The decortication reduces rancidity by removing outer bran layers rich in lipoxygenase enzymes and free lipids, minimizing oxidation while preserving nutritional quality.

2.9.2. Thermal treatments

In the past few years, thermal techniques have mostly been used on pearl millet for shelf-life extension. Dikkala et al. (2018) investigated the impact of heat treatment on PMF using an electric rotary dryer. The process was carried out at 150–170°C for 1.5 min at 300 rpm. This thermal treatment effectively reduced fungal contamination, thereby enhancing the microbial stability of the flour for up to 60 days. The application of high temperatures likely contributed to enzyme inactivation and moisture reduction, which are key factors in prolonging shelf life.

Some studies explored roasting to extend the shelf life of PMF. Narayanan & Ravikumar (2017) explored the impact of roasting on the microbial quality of pearl millet varieties Dhanshakthi and Proagro. They conducted storage experiments on roasted samples under refrigeration (4 °C) and room temperature (27.5 °C) for up to 60 days. After roasting and up to 30 days of storage, no detectable bacteria were found in either the packed or unpacked samples, even at a dilution of 10^{-5} g/mL. However, by day 45, the unpacked, refrigerated Dhanshakthi sample exhibited a total bacterial count of 5.30 log CFU/g, and by day 60, packed samples of both varieties stored at 27.5 °C reached 5.47 log CFU/g. These results suggest that microbial stability is maintained for up to 30 days in unpacked conditions and up to 45 days in packed, refrigerated conditions, beyond which microbial counts exceed levels typically considered acceptable for quality and safety. Therefore, under the tested conditions, pearl millet flour can be safely stored for these durations without significant microbial deterioration. Additionally, Proagro (packed) showed the highest reduction in FFA (78.8%) after 60 days under refrigeration. Dry roasting effectively deactivated lipolytic enzymes, minimizing fat triglyceride breakdown, with no change in peroxide value for the first 45 days. Both varieties experienced nutrient loss over time, with iron reduction ranging from 48% (Proagro-refrigerated) to 77% (Dhanshakthi-unpacked), likely due to mineral leaching or degradation from heating. Zinc content remained relatively stable, while protein and fat content

declined (22.9% and 4.3% in Proagro, 18% and 4.5% in Dhanshakthi) over 60 days. Padmaja et al. (2023) explored the impact of roasting (150 °C for 30 min) followed by modified atmospheric packaging (nitrogen gas flushed into the packaged flour) on extending the shelf life of PMF. Modified atmospheric packaging alters the package's gas composition, helping preserve the product. They found a maximum shelf-life (60 days) for the HHB67 variety under modified atmospheric packaging with LDPE, HDPE, and metalized laminated pouches. Alcoholic acidity refers to the accumulation of FFA and other acidic degradation products formed primarily through lipid oxidation and the enzymatic activity of lipase and lipoxygenase. Elevated alcoholic acidity can lower the flour's pH, creating conditions that may favor microbial growth.In the study, the treated sample exhibited a smaller increase in alcoholic acidity over time, rising from 0.19 to 0.66 g/100 g, compared to the control, which increased from 0.23 to 1.00 g/100 g. This suggests that the treatment effectively slowed the accumulation of acid-forming compounds. After 60 days, the treated samples also showed a significant reduction in FFA and peroxide values—by approximately 2-fold and 10–15-fold, respectively, compared to the control. These improvements in acidity and lipid stability may contribute to reduced microbial growth by limiting the availability of nutrients and creating a less favorable environment for spoilage organisms. Hydrothermal treatment is an effective method for extending the shelf life of PMF. It involves soaking the grains in water followed by controlled drying, which helps in enzyme inactivation and moisture regulation. Jalgaonkar et al. (2016) studied the effect of roasting and hydrothermal treatments with different storage conditions on the shelf life of pearl millet grains. Roasted flour has a shelf life of 2 to 3 days under ambient conditions of 30 °C. Hydrothermally treated flour had 30 and 45 days of shelf life. During the 60-day storage period, the fat acidity levels in PMF exhibited varying increases based on the type of treatment applied. Roasted flour, treated at 110 °C for 60 s,

experienced a 19.2-fold increase in fat acidity during ambient storage. In contrast, flour that underwent hydrothermal treatment (boiling for 15 min) showed a more moderate increase in fat acidity, rising by 58% during ambient storage. The slow increase in fat acidity is the desirable characteristic. Interestingly, the phytic acid content in untreated, roasted, and hydrothermally treated flour was 579 mg/100 g, 527 mg/100 g, and 600 mg/100 g (ambient storage), respectively. Trypsin inhibitor levels for treated, roasted, and hydrothermally treated flour were 8.73, 8.26, and 8.62 TUI/mg (ambient conditions), respectively. Heat treatments disrupted protease inhibitors, thereby improving protein digestibility.

2.9.3. Nonthermal treatment

There is limited study on exploring the shelf life of PMF while employing a nonthermal technique. Dikkala et al. (2018) explored irradiation as a nonthermal method to improve the microbial safety of PMF. Doses of 1 and 2.5 kGy were applied to evaluate their efficacy in reducing fungal contamination. Irradiation inhibited microbial growth without heat, preserving the flour's functional and nutritional properties for up to 60 days.

2.9.4. Addition of antioxidants

Abdalgader et al. (2019) examined the shelf life of two Sudanese pearl millet varieties, Ashana and Hreahry, treated with butylated hydroxyl toluene (BHT: 0.02%) and ascorbic acid (AA: 0.5%). Samples were stored in polyethylene bags at 37 °C and analyzed for FFA, peroxide value, and fat acidity over 90 days. Untreated Ashana cultivar FFA increased from 0.46% to 3.03%, while the level in the Hreahry cultivar increased from 0.3% to 3.1%. Peroxide values increased similarly. Fat acidity rose sharply, reaching 186.5 mg KOH/100 g for Ashana and 183.4 mg KOH/100 g for Hreahry variety. Both BHT and AA extended shelf life by up to 30 days at 37 °C. The lipid-rich nature of PMF might favor BHT's lipid-soluble properties, explaining its superior performance

over water-soluble antioxidant AA in preventing FFA formation, peroxide value increase, and fat acidity during storage.

2.9.5. Combined thermal and/or nonthermal treatments

Combining thermal and/or nonthermal methods can reduce microbial load and extend the shelf life.As previously described, Dikkala et al. (2018) applied a combined heat and irradiation treatment to PMF. While this approach achieved a 95.6% reduction in fungal contamination, storage studies revealed that microbial counts progressively declined up to 60 days but returned to near-initial levels by day 90. This indicates that although the treatment is effective in the short term, its efficacy diminishes over extended storage. Further research is needed to understand the factors contributing to microbial resurgence and to refine processing parameters for sustained shelf stability. Vinutha et al. (2022) concluded that combined treatment involving soaking for 1 h followed by thermal treatment at 100 °C for 15 min at 15 psi and infrared treatment using four 150 W NIR lamps operating at 240 V for 5 min, extended the shelf life of the grain up to 90 days. The untreated sample showed a shelf life of less than one week. The comprehensive peroxide value was consistently lower in the treated sample than the untreated sample throughout the storage period. The reduced peroxide value of the treated sample relative to the untreated sample was 85.1% on day 0, 90.7% on day 60, and 66.4% on day 90. While the reduction percentage declined slightly by day 90, the treated sample still maintained substantially lower peroxide values than the untreated sample, indicating improved oxidative stability throughout the storage period.

2.9.6. Comparing the shelf life of PMF from various techniques

Various thermal and nonthermal treatments have been explored to extend the shelf life of PMF, each with varying degrees of effectiveness in controlling microbial growth, rancidity, and nutrient degradation. Among the thermal treatments, roasting at 150 °C for 30 min extended the shelf life

to 90 days, outperforming other thermal methods like microwave heating (30 days) and dry heat treatment (30 days). Hydrothermal treatments, including soaking followed by controlled drying, have also demonstrated prolonged storage stability by reducing enzymatic activity. However, hydrothermal treatment compared to roasting resulted in a lower reduction in fat acidity and peroxide values, indicating a moderate impact on oxidative stability. Nonthermal treatments, such as irradiation, offer an alternative approach by effectively reducing microbial load without the heat-induced degradation of nutrients. Doses up to 2.5 kGy successfully suppressed fungal growth for 60 days, though the efficacy diminished after 90 days. This suggests that irradiation alone may not be sufficient for long-term preservation and is best used with thermal processing. Combined treatments incorporating thermal and nonthermal approaches have shown superior effectiveness in extending shelf life and maintaining flour quality. Heat treatment followed by irradiation resulted in a 95.6% reduction in fungal contamination, ensuring microbial safety for 60 days. Similarly, Vinutha et al. (2022) demonstrated that a combination of hydrothermal and near-infrared treatments extended the shelf life to 90 days.

While thermal techniques generally provide greater enzyme inactivation and microbial control, they also contribute to varying levels of nutrient degradation, particularly for heat-sensitive compounds like iron and protein. Nonthermal methods preserve nutritional quality but may be less effective in suppressing lipid oxidation over extended storage periods. Therefore, selecting a suitable preservation technique should be guided by the desired shelf life, nutrient retention priorities, and storage conditions. Future studies integrating multiple treatments and optimizing processing parameters could further enhance the storage stability of PMF while minimizing nutrient losses.

2.9.7. Principal component and correlation analysis

Multivariate data analysis was conducted to assess the impact of various factors on the storage stability of PMF after different treatments. These factors include grain variety, treatment type and conditions (such as temperature and time), and storage conditions (such as packaging material, nitrogen flushing, and temperature). The treatments were categorized into decortication, thermal (roasting, hydrothermal-drying, and hydrothermal-infrared), and antioxidant-based methods. Dikkala et al. (2018) reported the highest shelf life of 90 days at 34 ºC for PMF treated with dry heating followed by irradiation. However, this treatment was identified as an outlier in the data analysis due to a significant amount of unreported data. Principal component analysis (PCA) extracted two major components, PC1 and PC2, accounting for 45% and 20.1% of the total variance, respectively, with eigenvalues of 4.49 and 2.11 (Figure 2.4a). The PCA biplot reveals three major clusters: decorticated samples from Goswami et al. (2024), antioxidant-treated PMF from Abdalgader et al. (2019), and decorticated PMF from white and green varieties (Babiker et al., 2018). Hydrothermally treated samples exhibit significant variation due to subsequent drying or near-infrared (NIR) exposure. In contrast, roasted samples from Padmaja et al. (2023) appear scattered due to differences in storage temperatures (27 °C and 4 °C) and the effect of nitrogen flushing. Treatments like hydrothermal-infrared (Hyd-IR) and hydrothermal-drying (Hyd-Dry) show the most substantial impact on shelf life and quality, contributing to greater reductions in FFA and peroxide value. In contrast, decortication and antioxidant treatments demonstrate a moderate influence, primarily affecting enzymatic activity and oxidative stability.

The correlation plot (Figure 2.4b) highlights the relationships between different factors influencing the stability of PMF. Treatment temperature and time exhibit a strong positive correlation (0.79), indicating that higher time and temperatures are often associated with longer shelf life. Similarly, treatment temperature strongly correlates with shelf life (0.77), suggesting that higher roasting

temperatures improve PMF stability. However, treatment type negatively correlates with treatment temperature (-0.96) and treatment time (-0.82), implying that certain treatment methods involve lower temperatures and shorter durations. Storage temperature negatively correlates with shelf life (-0.32) and FFA content (-0.61), suggesting that higher storage temperatures may accelerate lipid degradation. Nitrogen flushing exhibits a moderate positive correlation with shelf life (0.31), indicating its role in extending storage stability. Interestingly, FFA strongly correlates with peroxide value (0.69), reflecting the interplay between lipid oxidation and rancidity development. These correlations provide valuable insights into optimizing treatment and storage conditions for enhancing the shelf life of PMF.

Table 2.11: Shelf life of pearl millet flour after various treatments.

Treatment	Conditions	Variety	Packaging	Storage Temp (ºC)	Shelf life (day)	FFA reduction (%)	PV reduction (%)	Other observations	Reference
Hydrothermal + Infrared	Soaking for 1 h at 30 ºC water + NIR exposure at 40 ºC/15 min with 480 W/m^2	Dhanshakthi	LLDPE	30	90	67.8	66.4	-Lipase activity decreased by 12.8 after soaking -NIR treatment inactivated 31.6% lipase	Vinutha et al. (2022)
Heat treatment + Irradiation	150-170 °C for 1.5 min in a rotary dryer + 2.5 kGy	NR	HDPE	34	90	NR	NR	-95.6% reduction in fungal contamination	Dikkala et al. (2018)
Roasting	Medium flame ~180 ºC for 10 min	Dhanshakthi	HDPE	27	60	2.1	0	- Moisture gain was 49-53% for Dhanshakti and 22-31% for Proagro -Unpacked flour (both varieties) showed a 5.6 log cfu/g bacterial count after 60 days	Narayana & Ravikumar (2017)
		Dhanshakthi	HDPE	4	60	62.6	10		
		Proagro	HDPE	27	60	13	0		
		Proagro	HDPE	4	60	60.8	20		
Decortication	Modern dehuller	White	LLDPE	25	60	NR	NR	-74 & 74.7% phytate reduction in white & green varieties, respectively	Babiker et al. (2018)
		Green	LLDPE	25	60	NR	NR		
Roasting	150 ºC for 30 min in hot air overn	HHB67	LDPE	27	60	13.6	24.5	-FFA changed by 45,7, & 5% for untreated HHB67, MPMH21, & Dhanshakti, respectively at 27 ºC/60 days - Lipase activity changed by 75,10, & 21% for untreated HHB67, MPMH21, & Dhanshakti, respectively at 27 ºC/60 days	Padmaja et al. (2023)
		HHB68	HDPE	27	60	16.3	29.4		
		HHB69	Met-lam	27	60	19	31.5		
		HHB70	LDPE+N_2 flush	27	60	28.2	30.1		
		HHB71	HDPE+N_2 flush	27	60	28.6	34.9		
		HHB72	Met-lam+N_2 flush	27	60	33.2	40.4		
		HHB73	LDPE	4	60	82	35.6		
		HHB74	HDPE	4	60	82.7	38.5		
		HHB75	Met-lam	4	60	84	43.1		
		HHB76	LDPE+N_2 flush	4	60	85.4	45.2		
		HHB77	HDPE+N_2 flush	4	60	86.3	48		
		HHB78	Met-lam+N_2 flush	4	60	87.7	52.7		

Roasting	110 ºC for 1 min within twin screw extruder	PC443	LDPE	30	45	6.5	NR	- Rapid moisture absorption occurred during storage -10% reduction in phytate after hydrothermal treatment	Jalgaonkar et al. (2016)
Hydrothermal + Drying	Soaking for 15 min at 100 ºC water + tray drying at 60 ºC/2 h	PC443	LDPE	30	45	93.1	NR		
Antioxidants	0.02% BHT	Ashana	LLDPE	37	30	34.2	33.8	-Ashana and Hreahry varieties had 5.12 & 5.43% fat, respectively	Abdalgader et al. (2019)
	0.5% AA	Ashana	LLDPE	37	30	32.5	25.9		
	0.02% BHT	Hreahry	LLDPE	37	30	42.7	28		
	0.5% AA	Hreahry	LLDPE	37	30	46.3	22.5		
Decortication	6.1-10% loss	PC 433	LLDPE	25	10	50	51.5	-71.9% decrease in lipase activity for GHB732 -58.3% decrease in acid value for GHB732 -45.7% reduction in total phenolics for HHB2226	Goswami et al. (2024)
		HHB67-Imp	LLDPE	25	10	58.3	20		
		GHB732	LLDPE	25	10	58.3	25		
		HHB226	LLDPE	25	10	58.3	31.3		
		Dhanshakti	LLDPE	25	10	65.4	33.3		
		MPMH17	LLDPE	25	10	55.1	30		
		86M01	LLDPE	25	10	55.2	35.9		
		GHB744	LLDPE	25	10	50	50		

Temp, temperature; FFA, free fatty acid; PV, peroxide value; NIR, near-infrared; BHT, butylated hydroxytoluene; AA, ascorbic acid; LDPE, low-density polyethylene; HDPE, high-density polyethylene; Met-lam, metalized laminated film; LLDPE, linear Low-density polyethylene; NR, not reported.

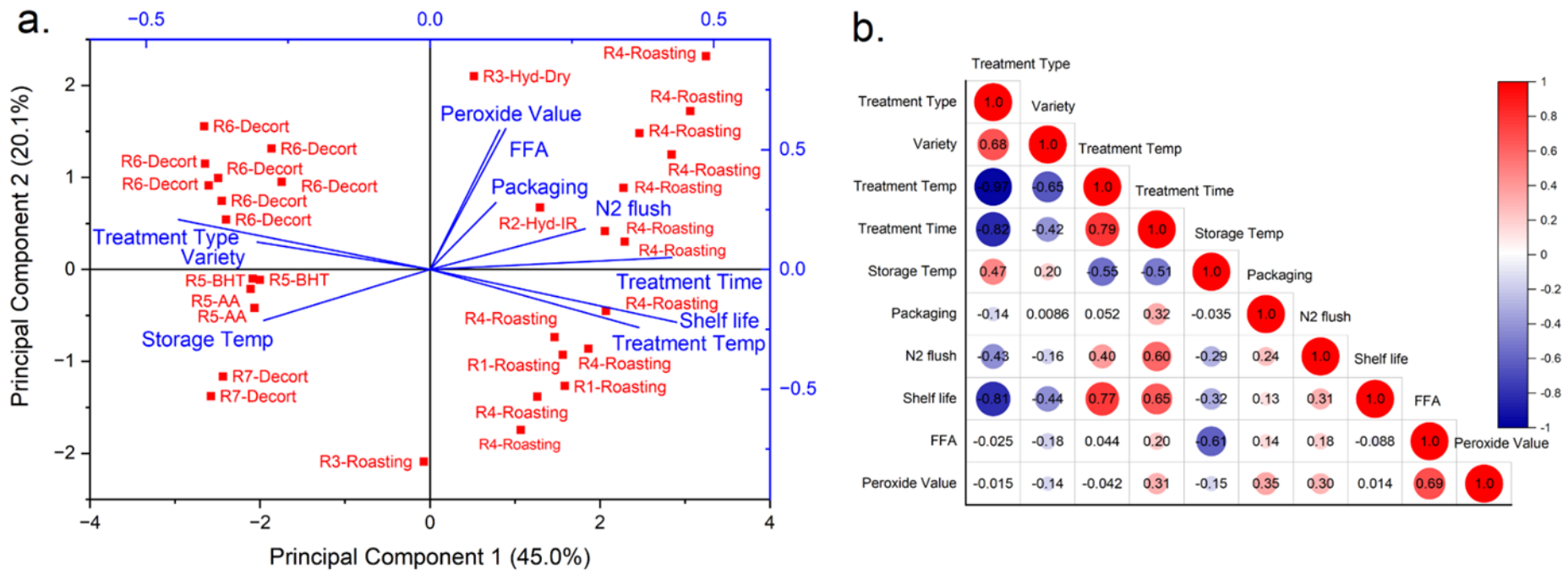

Figure 2.4: (a) Biplot and (b) correlation plot generated from principal component analysis (PCA) describing the impact of various factors on the shelf life of pearl millet flour after a set of different treatments. Nomenclature in biplot: R1, Narayana & Ravikumar (2017); R2, Vinutha et al. (2022); R3, Jalgaonkar et al. (2016); R4, Padmaja et al. (2023); R5, Abdalgader et al. (2019); R6, Goswami et al. (2024); R7, Babiker et al. (2018); Decort, decortication; Hyd-Dry, hydrothermal+drying; Hyd-IR, hydrothermal+infra red; BHT, butylated hydroxytolune; AA, ascorbic acid; Temp, temperature; FFA, free fatty acid.

2.10. Efficacy and Industrial Feasibility of the Processing Techniques for PMF

Comparing various treatments for pearl millet, such as fermentation, decortication, thermal, and nonthermal treatments, allows us to evaluate their effectiveness, efficiency, and suitability in achieving an optimal processing method. Moreover, getting a common base for the comparison is difficult. Fermentation reduces antinutrient properties by promoting the growth of beneficial microbes like lactic acid bacteria, which helps to break antinutrients such as phytic acid and tannins; cold plasma treatment enhances the functional properties by generating ROS, which interacts with the surface of the flour and modifies its structure. Hydrothermal and infrared treatments help enhance PMF's shelf life by inactivating spoilage enzymes. However, hydrothermal treatment compared to roasting resulted in a lower reduction in fat acidity and peroxide values, indicating a moderate impact on oxidative stability. The amount of energy needed for the treatment will also vary depending on the conditions. An optimal processing method for PMF should ensure microbial safety, retain its nutritional and functional quality, and provide enzymatic stability and longer shelf life. Various thermal and nonthermal treatments have been studied for their effectiveness in extending the shelf life of PMF. Among the thermal methods, roasting at 150 °C for 30 min extends shelf life up to 90 days, outperforming microwave heating (30 days) and dry heat treatment (30 days). In contrast, nonthermal methods like irradiation effectively suppress microbial contamination without nutrient loss, with doses up to 2.5 kGy preventing fungal growth for 60 days. However, irradiation alone may not suffice beyond 90 days, making combined approaches more effective.

Comparing all techniques based on a single parameter may not comprehensively evaluate efficiency. Instead, we must consider the optimal conditions across all parameters to determine the most efficient treatment for pearl millet to extend its shelf life and enhance its nutritional

properties. For instance, fermentation improves the protein content, reduces the antinutrient properties, and enhances the bioactive compounds in pearl millet flour (Chinenye et al., 2017; Choudhary et al., 2023). It is a time-consuming but more energy-efficient method compared to thermal treatments. In contrast, various thermal methods, such as roasting, blanching, and dry heat treatment, are commonly applied to pearl millet. Roasting effectively reduces microbial load and increases iron content, but it may also lead to nutrient loss (Obadina et al., 2016; Bhati et al., 2016). However, thermal treatments like roasting typically require higher energy input.

Cold plasma treatment has the potential to target pathogens and reduce antinutrients while preserving nutrients in PMF. However, its scalability for large-scale industrial applications, particularly in flour milling environments, is limited by high voltage requirements, safety hazards, and energy consumption. While some studies suggest cold plasma as a cost-effective method, its practicality remains uncertain. The effectiveness of cold plasma treatment largely depends on the plasma type, gas composition, and operational settings. For instance, Charu et al. (2023) utilized a dielectric barrier discharge low-pressure air plasma system at 2 kV for 5 min, whereas Lokeswari et al. (2021) employed an atmospheric pressure jet plasma system at 40–45 kV for 5–15 min, using atmospheric air. Sarkar et al. (2023) conducted experiments with a multipin discharge atmospheric cold plasma reactor at 20–30 kV for 10–20 min. These variations impact the physicochemical changes in PMF; optimal conditions remain unclear. Further research is needed to assess the feasibility of cold plasma treatment at an industrial scale and compare its efficacy with alternative cost-effective technologies better suited for large-scale milling operations. Fermentation is also beneficial for PMF in terms of nutrient retention and microbial reduction, offering a balanced approach to efficiency, cost-effectiveness, and energy consumption. Studies have demonstrated that combined thermal and nonthermal methods (heat treatment + irradiation and hydrothermal +

near-infrared treatments) yield superior results. Therefore, optimizing a hybrid processing strategy is crucial for balancing shelf life, nutrient retention, and industrial feasibility. Further research into optimizing these techniques for large-scale applications on nutritional properties and cost efficiencies in specific grains would benefit practical implementation in food processing industries.

2.11. Strategy to Design a Process for Pearl Millet Flour

Microbial safety, enzymatic stability, and nutritional quality are the key factors in designing a process for PMF. High microbial counts can make PMF unsafe, leading to foodborne illnesses, while enzymatic activity is the primary cause of rancidity in the flour. Manufacturers, consumers, and retailers each prioritize different aspects of PMF quality. Manufacturers prioritize microbial safety to ensure product stability and compliance with food safety regulations. Consumers seek a nutritionally rich product with minimal antinutritional factors, while retailers focus on maintaining product appearance and preventing off-flavors caused by rancidity. While these goals are generally aligned, they can sometimes conflict during the design and processing of PMF. Intensive processing methods that ensure microbial safety can lead to nutrient degradation. For instance, thermal treatments designed to kill harmful microorganisms might also result in the loss of heat-sensitive nutrients. Similarly, processes that prevent rancidity (such as high-heat treatments) can further compromise nutrient retention. Therefore, optimizing processing conditions to balance all three factors is the key challenge. Microbial safety and enzymatic stability must be prioritized to extend shelf life and maintain product quality. At the same time, nutrient retention should be preserved by avoiding excessive heat or treatment time.

The existing strategy for processing PMF ensures microbial safety; however, enzymatic stability in the products attracts much less attention. Enzymatic stability refers to inhibiting the activity of

enzymes that cause spoilage and quality degradation in PMF. Key enzymes like lipase, LOX, PPO, and POD can lead to rancidity, off-flavors, discoloration, and nutrient loss. Thus, achieving the inactivation of these enzymes is essential for preserving the nutritional value, shelf life, and sensory qualities of the flour. The goal is to inactivate spoilage enzymes (>99% inactivation), particularly lipase while minimizing the loss of essential nutrients. An ideal process design should integrate enzymatic stability alongside microbial safety, nutrient retention, and anti-nutrient reduction, with lipase as a key indicator for optimization.

Antinutritional components are also important factors that negatively influence the nutritional properties of PMF. Reducing the antinutrients will improve the nutritional quality and bioavailability of foods. The average phytate intake varies by country, with the US and UK ranging between 631 and 746 mg/day, Finland at 370 mg/day, Italy at 219 mg/day, and Sweden at 180 mg/day (Jalgaonkar et al., 2016). Achieving antinutrients at this optimal concentration will prevent micronutrient deficiencies, improve digestibility, and enhance palatability and acceptance of pearl millet-based products. On the other hand, reducing the microbial load will prevent foodborne illness. The target for microbial inactivation can be set as a 5-log cycle for aerobic mesophilic microflora (Christelle et al., 2021). Therefore, the process for PMF must be optimized to achieve a 5-log reduction in the most resistant pathogen count, ensuring microbial safety. Additionally, the process should be designed to maximize nutrient retention while minimizing antinutrient levels. There is limited research on the enzyme activity and microbial safety of the PMF.

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Chapter -3

Inactivation of Enzyme Activity in Pearl Millet Flour using Atmospheric Cold Plasma and Steam Treatments: Kinetics and Impact on Its Antinutritional and Compositional Properties

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Abstract

Pearl millet flour (PMF) has a well-balanced protein content with fat, gluten-free, and low glycemic index, making it a substitute diet for diabetes and celiac disease. However, PMF spoils quickly due to enzymatic rancidity, thus limiting its shelf life, and anti-nutrient factors, such as tannins and phytic acid, also limit its usage. This study aims to inactivate quality deteriorating enzymes, viz. lipase, lipoxygenase, polyphenol oxidase (PPO), and peroxidase (POD) by using cold plasma (5-25 kV; 2-10 min). Cold plasma treatment at 25 kV/8 min, led to 95.9 %, 99.2 %, 94.8 %, and 100 % inactivation of lipase, lipoxygenase, peroxidase, and PPO activity, respectively. Cold plasma-induced enzyme inactivation followed n th-order kinetics, such as 1.55, 1.35, 1.15, and 1.05 for lipase, peroxidase, lipoxygenase, and PPO, respectively. Steam treatment at 100 °C/4 min inactivated lipase, lipoxygenase, peroxidase, and PPO by 97.9, 98.8, 97.6, and 100 %, respectively. The PMF showed 24.0 g/hg and 19.5 g/hg reduction in phytic acid and 77.7 g/hg and 16.9 g/hg reduction of tannin content at 25 kV/8 min of cold plasma treatment and steam treatment at 100 °C/5 min, respectively. Color, particle size, and proximate composition remained unaffected; a reduction in secondary oxidation products and minor modifications in the secondary structure of the protein were observed in both treatments. Additionally, cold plasma treatment enhanced the functional properties of the PMF. Cold plasma as an alternative to thermal treatment to inactivate enzymes and antinutrients while preserving compositional attributes of PMF.

Keywords: *Lipase, Lipoxygenase, Peroxidase, Polyphenol oxidase, Phytates.*

3.1. Introduction

Pearl millet (*Pennisetum glaucum*), a resilient Poaceae crop, is an important global food source. India is the leading producer, contributing 39% of global millet output in 2024-25 (12 million tons, of which 8.6 million tons are pearl millet), followed by Niger, China, and Nigeria (USDA FAS, 2025). Pearl millet flour (PMF) is nutrient-dense and rich in healthy fats (polyunsaturated with low cholesterol), crude fiber (1.3-2.8 g/hg), B vitamins (B2, B1, and B6), and essential minerals (Ca, Mg, P, K, and Na) (Naraharasetti et al., 2025; Nurmomade et al., 2024). Its polyunsaturated fats, omega-3 fatty acids, and essential amino acids (arginine, threonine, valine, leucine, and isoleucine) support the management of diabetes, cardiovascular disease, and arthritis (Charu et al., 2024). Compared to maize and other millets, PMF offers better digestibility, is naturally gluten-free, and has a relatively low glycemic index (55 for PMF vs. 70-104 for sorghum, kodo, and finger millet) (Lokeswari & Mahendran, 2022). Despite its nutritional potential, pearl millet remains underutilized due to post-harvest and processing challenges. Its fibrous pericarp contains anti-nutritional factors such as phytates, polyphenols, tannins, and fiber, which bind minerals like iron and zinc and reduce their bioavailability (Jha et al., 2015). Phytate, the main phosphorus store in plants, occurs in pearl millet at 179-306 mg/100 g (Boncompagni et al., 2018; Mayer et al., 2023). Tannins, present at ~1.4 g/hg also form complexes with proteins and minerals, reducing absorption and imparting astringency (Ahmad et al., 2018; Song et al., 2024).

Additionally, high lipase enzyme activity in pearl millet results in undesirable flavors and discoloration in final products, limiting its use to traditional consumers (Lokeswari & Mahendran, 2022). Rancidity in pearl millet flour occurs via two major routes: enzymatic and nonenzymatic processes. Enzymatic rancidity, primarily caused by lipase, lipoxygenase (LOX), polyphenol oxidase (PPO), and peroxidase (POD), produces unpleasant odors. The fat content (3-8 g/hg) is

the main factor behind the rapid increase in hydrolytic acidity in ground millet. The lipase enzyme is primarily responsible for lipid breakdown and the generation of free fatty acids (FFA). Lipase catalyzes the hydrolysis of triacylglycerol (TAG) into diacylglycerols, monoglycerols, glycerol, and FFAs. These FFAs serve as substrates for LOX, which produces lipid hydroperoxides, the primary oxidative products. LOX promotes the oxidation of polyunsaturated fatty acids, resulting in hydroperoxides (Narayanan & Ravi Kumar 2017). LOX generates highly reactive molecules that impact the organoleptic properties and color of the flour. POD and PPO react with lipid hydroperoxides to produce reactive oxygen species (ROS), which further attack FFAs to produce a greater concentration of lipid hydroperoxides. These hydroperoxides undergo non-enzymatic oxidation, forming secondary oxidation products like aldehydes and ketones, which have pungent flavors and lead to rancidity during storage (Goswami et al., 2023).

Limited research has examined the inactivation of enzyme activity and reduction in antinutrient factors in PMF through processing. Vinutha et al. (2022) found that a combined hydrotreated-hydrothermal-near-infrared treatment significantly reduced the activities with lipase decreasing by 47.8 %, LOX by 84.8 %, peroxidase by 98.1 %, and PPO by 100 %. Additionally, there was a decrease of 67.8 g/hg in FFAs and 66.4 g/hg in peroxide content. Yadav et al. (2012) reported that microwave heating of pearl millet grain (18 g/hg moisture, 900 W for 80 s, final temperature 107.6–110 °C) significantly reduced lipase activity by 88.5 %. Tiwari et al. (2014) found that heating PMF at 110 °C for 60 s significantly reduced lipid acidity by 44.6 g/hg and FFAs by 84.6 g/hg. Goswami et al. (2024) investigated the impact of decortication on grain rancidity, noting that low-level decortication reduced LOX activity, comprehensive peroxide value, and malondialdehyde content by 11.2 %, 12.5 %, and 9.7 %, respectively. Thermal treatment leads to the degradation of heat-sensitive nutrients and vitamins, apart from the alteration of the starch and

functional properties of the material (Sarkar et al., 2023). Decortication leads to the partial inactivation of the enzyme activity. Due to drawbacks in the current processing technologies, there is a need for novel non-thermal technologies to inactivate the enzyme activity and enhance the shelf life of pearl millet flour without changing nutrient properties.

Cold plasma, a non-thermal technology that contains various reactive molecules, such as free radicals, ions, atoms, and electrons. These energetic particles interact with the structural, molecular aspects, and biological components of food (Doddabematti Prakash et al., 2023; Christaki et al., 2025). Some studies have explored the influence of cold plasma on various nutritional and functional attributes of PMF. Sarkar et al. (2023) found the impact of atmospheric cold plasma (ACP) treatment (20-30 kV for 10-20 min) on pearl millet that enhanced starch gelatinization at 30 kV for 20 min. In another study, Lokeswari et al. (2021) studied ACP treatment on pearl millet (40-45 kV for 5-15 min), which improved color intensity, pasting, and nutritional properties. Kaur & Annapure (2023) found that cold plasma treatment at 230 V/30 min significantly modified the pearl millet starch attributes, leading to a reduction in turbidity and pH values. Gnananethri et al. (2024) studied the effect of cold plasma treatment on bioactive, functional properties of PMF at 25-35 kV/10 min. Lokeswari & Mahendran (2022) investigated the effects of 24 L air plasma bubbling on pearl millet at 200 V. This treatment improved the flaking and puffing characteristics while notably influencing the crispiness, hardness, color, and moisture content.

Cold plasma treatment helps to reduce the antinutritional properties and microbial load of pearl millet flour. For instance, Lokeswari et al. (2021) examined the effects of 120 L plasma-processed air bubbling and soaking on pearl millet at 180 V, lowering 21.6 g/hg phytic acid in the sample. In a related study, Charu et al. (2024) found that cold plasma treatment (2 kV/5 min) led to significant changes in fat content, vitamin B-complex, antioxidant activity, and reduced microbial counts in

pearl millet flour. Additionally, it led to a reduction of 82 g/hg tannins and a 57 g/hg phytic acid. However, no study has focused on enzyme inactivation and kinetics during cold plasma and thermal treatments of PMF. The information from kinetics is obligatory when designing the process for obtaining an enzymatically stable PMF with an extended shelf life.

Some of the studies explored the enzyme activity and impact of antinutrient factors through the cold plasma in other millets. For instance, Jaddu et al. (2022a) studied the functional properties of kodo millet flour using a multipin plasma system at 10-20 kV for 10-30 min. Sonkar et al. (2023) treated millet starch in a plasma reactor at 10-30 kV for 10-30 min. Both studies found that cold plasma enhanced the hydration and structural attributes of kodo millet starch by improving oil, water absorption, and swelling capacity. Chauhan et al. (2023) studied the impact of pin-to-plate ACP on various properties of millet starch at 170-230 V for 5-15 min. Plasma-induced depolymerization reduced amylose content, increasing paste clarity but reducing viscosity.

The study aims to explore the impact of cold plasma treatment on the inactivation kinetics of spoilage-causing enzymes (lipase, LOX, POD, and PPO) in PMF. Steam treatment is also assessed to compare with the cold plasma treatment. The minimum treatment condition required for 90 % inactivation of all the quality-deteriorating enzymes in PMF was determined for both thermal and non-thermal treatments. Further, antinutritional factors (phytate and tannin content), bioactive content (total phenolics, flavonoids, antioxidant capacity), lipid oxidation products, functional properties, protein structure, physical characteristics such as particle size and density, along with proximate composition, water activity, and amount of damaged starch, were evaluated between the thermal and non-thermal-treated PMF.

3.2. Materials and Methods

3.2.1. Whole pearl millet flour

The pearl millet (*Pennisetum glaucum*), sourced from Ancient Wisdom Organic Bajra Grain, Mumbai, Maharashtra, India, was used in this study. Dhanshakti variety is used throughout the study, which has the highest enzyme activity of lipase, LOX, PPO, and POD (67, 199, 0.09, and 110 mMol substrate.min^{-1}. (mg/protein) respectively) compared to all other pearl millet varieties (Vinutha et al., 2022; Naraharasetti et al., 2025). The sample was sourced while ensuring that it came from the same batch of Dhanshakti variety.1 kg of pearl millet grains was passed through the hammer mill, which runs at 3,000 rpm (GlenMills Inc., Clifton, NJ, USA type: SK300;1100 W, 5 A) for 30 min. The flour was obtained as whole flour without separation of the bran, germ, and shorts. The yield obtained through the milling was 88%.

3.2.2. Experimental design

For the cold plasma treatment of PMF, a full factorial design (5^2) was employed. The two independent variables were root mean square (rms) voltage (V_{rms}, kV) and treatment duration (t, min). The V_{rms} was tested at 5 variable conditions (5, 10, 15, 20, and 25 kV) while exposed to 5 variable conditions of treatment time (2, 4, 6, 8, and 10 min). Cold plasma studies were carried out in 25 conditions of Voltage and treatment time. In a separate set of experiments, PMF was subjected to steam treatment at 100°C for different times of 1, 2, 3, 4, and 5 min. All the experiments throughout the study were conducted in triplicate. The activities of lipase, lipoxygenase, peroxidase, and polyphenol oxidase in the PMF were measured after each treatment. Furthermore, the flour's antinutrient content and bioactive compounds were tested under optimum conditions following the cold plasma and steam treatment, after kinetic modeling. The cold plasma and steam treatments that led to more than 90 % enzyme inactivation of the most resistant enzymes

were selected for further analysis. This subsequent analysis focused on evaluating physical and chemical attributes, including particle size, density, water activity, proximate composition, damaged starch, antinutrient content, and bioactive compounds in the flour.

3.2.3. Cold plasma treatment

A multipin-plane plasma reactor (Model: LCP-MP, Ingenium Technologies, Odisha, India) operating in batch mode in atmospheric air conditions was utilized. This system can achieve up to 30 kV Voltage. It consists of an electric control panel, a step -up high–voltage transformer, and a cold plasma reactor. The power transformer (Model: LCP-LPS40) is built with stainless steel 304 with an epoxy glass holder. It features a custom-designed, oil-cooled step-up transformer with superior electrical insulation strength and high-breakdown voltage. The cold plasma power supply runs on a typical single-phase electrical supply (15 A,60 Hz, and 100–110 V) and incorporates a digital metering system. It is capable of delivering 12 mA of current and 40 kV voltage, ensuring effective operation by delivering 1200 W of power (Chakraborty et al., 2024).

Pearl millet flour weighing 5 g was carefully transferred to the petri dishes and evenly spread. The enclosed petri dish was positioned in the middle of the cold plasma equipment. A measurement of 1.5 cm was made of the vertical gap between the upper surface of the flour in the petri dish and the lower edge of the pin. The space between the upper surface of the flour to the lid was 0.9 cm. The Voltage was set to range from 5 to 25 kV in the control panel, with a duration of treatment ranging from 0 to 10 min. To achieve target voltages of 5, 10, 15, 20, and 25 kV, the system requires 17, 21, 26, 29, and 32 s, respectively. During the treatment duration, voltage varied ± 1 kV and current varied from 0.7 and 0.9 mA.

3.2.4. Steam treatment

PMF was placed in a stomacher bag, which was then sealed after removing as much air as possible. The autoclave (Tomy Seiko Co., Ltd., Tokyo, Japan, model: SS-325E) was used to process the sealed pouch operating at 100°C of steam. The bags were subjected to the steam treatment for the specified durations (1 to 5 min). Following the treatment, the bags were taken out, their surfaces were blotted, and then allowed to air-cool. After the treatment, the pouches cooled to room temperature (25 ± 2 °C), and the PMF was extracted for subsequent analysis.

3.2.5. Enzyme assay

Enzyme assays were performed for lipase, lipoxygenase, polyphenol oxidase, and peroxidase based on spectrophotometric assays expressed in terms of katal (kat/mol·s^{-1}). The kat value of enzymes is calculated based on the rate of product formation, extinction coefficient, and the reaction conditions of the enzyme assay. kat of lipase is *P*-nitrophenol released per minute at 37 ℃ in 15 minutes, and the extinction coefficient for the *P*-nitrophenol at 400nm. kat of lipoxygenase is formed conjugated diene from linoleic acid oxidation per minute at 30 ℃ for 2.5 min. The extinction coefficient for conjugated dienes at 234 nm. kat of peroxidase is the oxidized ABTS formed per minute in an incubation time of 3 minutes. ABTS extinction coefficient taken at 405nm. kat of the polyphenol oxidase is the quinone formed per minute at 410nm, having a substrate solution made up of MOPS buffer and catechol. Lipase and lipoxygenase activity were assessed by using the following method Chakraborty et al. (2024).

3.2.5.1. Lipase - Lipase was extracted with distilled water from PMF through the vortex, shaken at room temperature for 30 min, and then centrifuged at 4500 × g for 15 min. Then, the extract was mixed with the Tris buffer (0.1 M, pH 8.2) and the substrate solution (prepared from 15.9 g p-nitrophenyl palmitate, 17 mg sodium dodecyl sulfate, 1 g Triton X-100, and 100 mL of deionized

water), followed by incubation at 37 °C for 15 min. Using a spectrophotometer, the absorbance was measured at 400 nm.

3.2.5.2. Lipoxygenase - PMF was extracted using phosphate buffer (pH 6.5), vortexed for 30 min and centrifuged at 5000×g for 15 min at 4 °C. Extract was mixed with substrate solution (made with 157 µL linoleic acid, 157 µL of tween-20, 5 mL of distilled water, and 194.7 mL phosphate buffer). NaOH was added to 1 mL of the mixture following 2.5 min incubation at 30 °C for 2.5 min. The absorbance was then measured using a spectrophotometer at 234 nm.

3.2.5.3. Polyphenol oxidase -This assessment for polyphenol oxidase was followed by Anderson and Morris et al. (2001). 1 mL of MOPS(1M) was combined with 19 mL of distilled water, and subsequently by the addition of 0.022 g of catechol to prepare the substrate solution. The substrate solution was added to the sample. At room temperature mixture was incubated for an hour through continuous shaking. After incubation, centrifugation was done at 1000 × g for 20 min. The absorbance was measured using a spectrophotometer at 410 nm.

3.2.5.4. Peroxidase -The assessment for peroxidase was followed by Almeida et al. (2019). The enzyme was extracted by adding the flour to 5 mL phosphate buffer (pH 5.0), followed by continuous shaking for 30 min. Centrifugation was carried out at 1000 × g for 10 min. ABTS (2,2-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid) and hydrogen peroxide were added to the extract, and the absorbance of oxidized ABTS was measured at 405 nm using a spectrophotometer.

3.2.6. Sensitivity analysis

Sensitivity analysis is a method used to determine how changes in input variables affect the output variables. Sensitivity analysis was conducted based on the voltage (S_v) and time (S_t) at 4 levels of the experimental design at 5 kV/2 min, 5 kV/8 min, 25 kV/2 min, and 25 kV/8 min. The sensitivity index is determined using eq (1) and (2) as described by Chakraborty et al. (2024). In the equation,

the standard deviation of Y values is denoted by σ, and Y is the inactivation of the enzymes, which is quantified based on the initial enzyme activity (A_i) and final enzyme activity (A) after the treatment.

$$Sv = \frac{1}{\sigma}\left[\frac{\sum y_{25kv}}{2} - \frac{\sum y_{5kv}}{2}\right] \quad (1)$$

$$St = \frac{1}{\sigma}\left[\frac{\sum y_{5\,min}}{2} - \frac{\sum y_{1min}}{2}\right] \quad (2)$$

3.2.7. Kinetic modeling

To analyze enzyme inactivation kinetics for each voltage and time level of cold plasma treatment, a primary assessment was conducted to evaluate both first-order (n = 1) and n^{th} order kinetic models. The first order (log-linear) equation is represented in eq (3) where A_i is the initial enzyme activity, A is the enzyme activity that remains after t min of the treatment, and k (min^{-1}) is the rate constant.

$$ln\left[\frac{A}{A_i}\right] = -kt \quad (n = 1) \quad (3)$$

eq (4) describes the n^{th} order kinetic model. A/A_i (%) represents the residual enzyme activity, and n is the inactivation order.

$$\frac{A}{A_i} = [1 + A_i^{n-1}(n-1)kt]^{\frac{1}{1-n}} \quad (n \neq 1) \quad (4)$$

To determine an inactivation order (n) across all the treatment voltage and time combinations, the sum of square errors (SSE) between the experimental and predicted residual enzyme activity values was minimized through an optimization algorithm designed by Chakraborty et al. (2015).

3.2.8. Phytic acid and tannin content

Phytic acid content of PMF was measured using the method by Shrivastava and Chakraborty (2018). The phytic acid content was expressed in equivalent phytic acid ($mg \cdot kg^{-1}$). The sample is extracted with the 3 g/hg trichloroacetic acid (TCA) solution through a vortex for 45 min. The

extract is added to the ferric chloride solution. Precipitate is produced by boiling the solution for 45 min in a water bath. The precipitate was dissolved in NaOH (1.5 mol equi/L). The quantification of phytic acid was measured by taking absorbance at 480 nm. Tannin content was determined using Garg et al. (2020). The amount of tannin content was stated as mg gallic acid equivalents·kg^{-1}.

3.2.9. Total phenolics, flavonoids and antioxidant activity

Total phenolic content (TPC) was measured through the following procedure by Siroha et al. (2016). The sample extracted with methanol (1:20) was centrifuged, and the extracted supernatant was used to determine TPC, total flavonoids, and antioxidant activity. For total phenolics, supernatant was added to the FC (Folin–Ciocalteu) reagent and allowed to stand for 5 min. The extract was subsequently combined with distilled water and sodium carbonate (7.5 g/hg). This solution was incubated for 90 min in the dark at room temperature. The concentration of the blue-colored complex was assessed using a spectrophotometer at 725 nm. TPC was represented in mg of gallic acid equivalent (GAE)·kg^{-1}. To estimate flavonoids, the supernatant was added to the distilled water and 5 g/hg sodium nitrite (Siroha et al., 2016). After adding the 1 M sodium hydroxide solution and 10 g/hg aluminum chloride to the mixture, it was allowed to stand for 5 min. At 510 nm, the mixture of absorbance was measured. Total flavonoids were measured in the catechin equivalent (mg CE·kg^{-1})

Antioxidant activity was tested using the method detailed by Chakraborty et al. (2015). DPPH (2,2-diphenyl-1-picrylhydrazyl) was incorporated into the extract and allowed to stand in the dark for 20 min. Quantification of the activity is done by measuring absorbance at 565 nm. Antioxidant activity was quantified in mg gallic acid equivalents·kg^{-1}.

3.2.10. Lipid oxidation products

Primary and secondary lipid oxidation products released during the steam and cold plasma treatment conditions in PMF were evaluated by using the free fatty acid content, peroxide value, and thiobarbituric acid reactive substance test (TBARS). Free fatty acid (FFA) content was measured gravimetrically by following the protocol of Jia et al. (2021). The peroxide value was determined using the titration method described by Kumar et al. (2021). The thiobarbituric acid reactive substance test was measured based on the malondialdehyde (MDA)/kg flour using the standard curve method described in Abeyrathne et al. (2021).

3.2.11. Secondary protein structure

FTIR Spectra of PMF were determined by a Perkin Elmer FTIR spectrometer (Spectrum 400, Universal ATR Sampling Accessories, PerkinElmer, Shelton, CT, USA) following the method by Pulivarthi et al. (2025). A total of 32 scans were conducted for each sample of PMF with intervals of 4 cm^{-1} in the range of 400-4000cm^{-1}. The spectrum data were analyzed using the Origin Pro 2023b software (OriginLab Inc., Northampton, MA, USA).

3.2.12. Functional properties of PMF

Emulsion capacity, water absorption, and oil absorption capacity were determined by following the method described by Pulivarthi et al. (2025) with slight modifications. In brief, 1 g of the sample was mixed with 10 mL of oil and 10 mL of water in separate centrifuge tubes for oil absorption and water absorption, respectively. The mixture was allowed to stand for 30 min at room temperature, then centrifuged at 3000 × g for 10 min. The supernatant was discarded, and the weight of the flour was measured to analyze oil and water absorption capacity. To determine emulsification capacity, 1.75 g of the flour was homogenized with 25 mL of deionized water for 30 s, followed by 25 mL of oil was added to the suspension and homogenized for another 30 s.

The resultant mixture was centrifuged at 1100 × g for 5 min. Emulsion capacity was measured based on the height of the emulsion layer.

3.2.13. Physicochemical properties

The moisture content of PMF was measured with the hot air oven technique (AACC Method 44-40.01). Protein and ash content in the PMF were evaluated using the combustion (AACC Method 46-30.01) and muffle furnace (AACC Method 08-01.01) methods, respectively. The particle size analysis of PMF was conducted using the Ro-Tap Sieve shaker (Hosokawa Micron, E20), ranging from 420 µm (US mesh no.40) to 53 µm (US mesh no.270). The bulk, tapped density, geometric mean diameter, and particle size were calculated using the method proposed by Pulivarthi et al. (2022).

3.2.14. Statistical analysis

Each experiment and analysis were performed in triplicate. The Origin Pro 2023b software (Origin Lab Corporation, Northampton, MA, USA) was used for the analysis of variance (ANOVA), non-linear curve fitting, and execution of Tukey's HSD test. Inactivation order and linear curve fitting were performed using Microsoft Excel (Microsoft Office system, USA).

3.3. Results and Discussion

3.3.1. Sensitivity of lipase, lipoxygenase, and peroxidase toward cold plasma treatment

The newly introduced sensitivity index is the relative change in the enzyme activity due to the changes in either 'RMS voltage' or 'exposure time'. The higher the sensitivity, the higher the influence of that variable, like voltage or time. The aim was to identify the most sensitive enzyme to cold plasma treatment. The common enzyme showing the least sensitivity toward both voltage and time would have been the resistant (target) enzyme for process design. The sensitivity indices with respect to voltage (S_v) for lipase, lipoxygenase, and peroxidase of cold plasma-treated PMF

were 1.692, 1.704, and 1.660, respectively. Voltage has a high influence on all enzymes. among all 3 enzymes, lipoxygenases are more affected by voltage. higher sensitive indices indicate greater sensitivity to the voltage. Lipase, lipoxygenase, and peroxidase had sensitivity indices for treatment time (S_t) of 0.140, 0.178, and 0.235, respectively (Table 3.1). Peroxidase has a higher sensitivity to time among the 3 enzymes. Treatment time has the least influence on the enzymes compared to the time duration. Polyphenol oxidase is more susceptible to cold plasma treatment. It is inactivated at only 5 kV/8 min. It is due to the copper-containing enzyme being more exposed and reactive to the oxidative damage from plasma-generated radicals. The tertiary structure of the PPO enzyme is made by electrostatic and hydrophobic interactions. Plasma-generated radicals lead to the formation of protein carbonyls and cause partial unfolding or conformational changes in the molecule. Disruption of the tertiary structure results in exposure of the copper active site, making it more accessible to oxidative damage. quaternary structure is dissociated through radical-mediated cleavage of non-covalent bonds and protein subunits (Laika et al., 2024). Cold plasma exposure inactivates the lipoxygenase enzyme more than lipase or peroxidase, based on the sensitivity analysis. Peroxidase is the most resistant enzyme because it has less sensitivity to cold plasma voltage (1.660) compared to the other enzymes, lipase and lipoxygenase. Peroxidase exists as dimers or tetramers and has multiple disulfide bonds, which provide strong covalent links to stabilize the structure and give resistance to the oxidative stress created by the cold plasma radicals. The presence of calcium-binding sites and a coordinated hydrogen bond network also contributes to the stability of the enzyme from the treatment (Bernardes et al., 2015). Peroxidase enzyme inactivation should be prioritized in the PMF. Surowsky et al. (2013) observed that POD is more resilient to treatment conditions than PPO after 240 s of cold plasma in model food systems. Lipoxygenase is more responsive than lipase in whole wheat flour, as found by Chakraborty et al.

(2024). The reduction in enzymatic activity is determined by the type of plasma equipment utilized, the feed gas, and the amount of power delivered (Sadhu et al., 2017). Enzymes resistance to cold plasma treatment depends on their structural characteristics. Enzymes respond differently to varying voltage levels based on their resistance, which is influenced by their molecular structure (Misra et al., 2016).

Table 3.1: Sensitivity index of lipase, lipoxygenase, and peroxidase in pearl millet flour inactivated by cold plasma treatment.

Treatment Condition		Residual Enzyme Activity (%)		
Voltage (kV)	Time (min)	Lipase	Lipoxygenase	Peroxidase
5	2	94.4 ± 2.3^{b}	97.7 ± 1.5^{c}	98.4 ± 1.5^{c}
5	8	85.4 ± 4.9^{a}	92.2 ± 2.9^{b}	91.2 ± 3.8^{b}
25	2	25.5 ± 3.9^{b}	22.3 ± 4.5^{b}	35.3 ± 2.7^{d}
25	8	4.1 ± 3.1^{a}	0.8 ± 2.1^{a}	5.2 ± 3.5^{a}
Sensitivity index based on voltage (S_v)		1.692	1.704	1.660
Sensitivity index based on time (S_t)		0.140	0.178	0.235

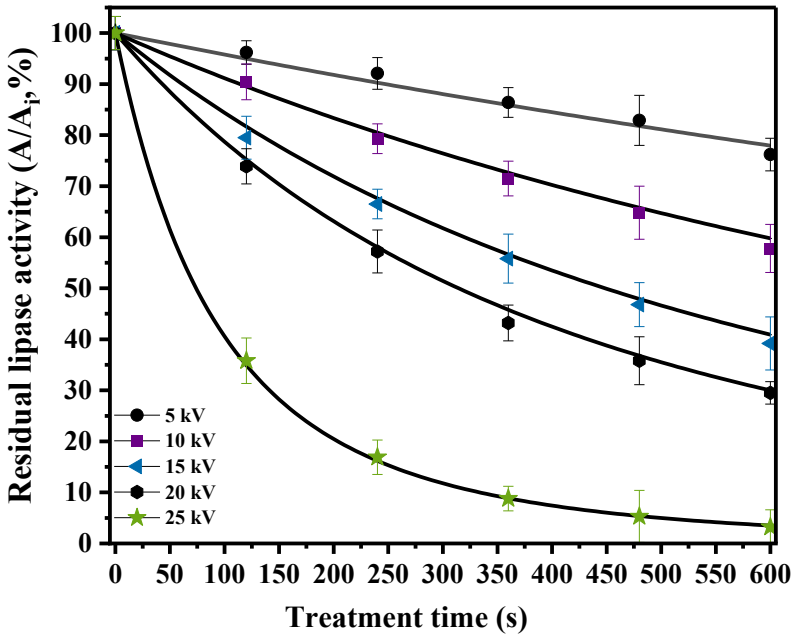
The dissimilar alphabets (a, b, & c) in the superscripts denote that the mean values are statistically different across the treatment conditions at $p < 0.05$.

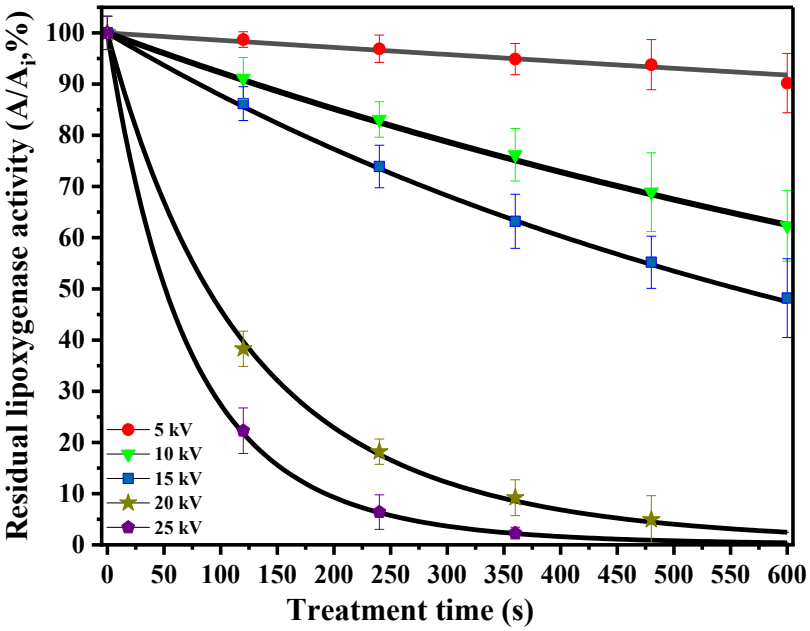
3.3.2. Influence of cold plasma treatment on the enzyme activity of pearl millet flour

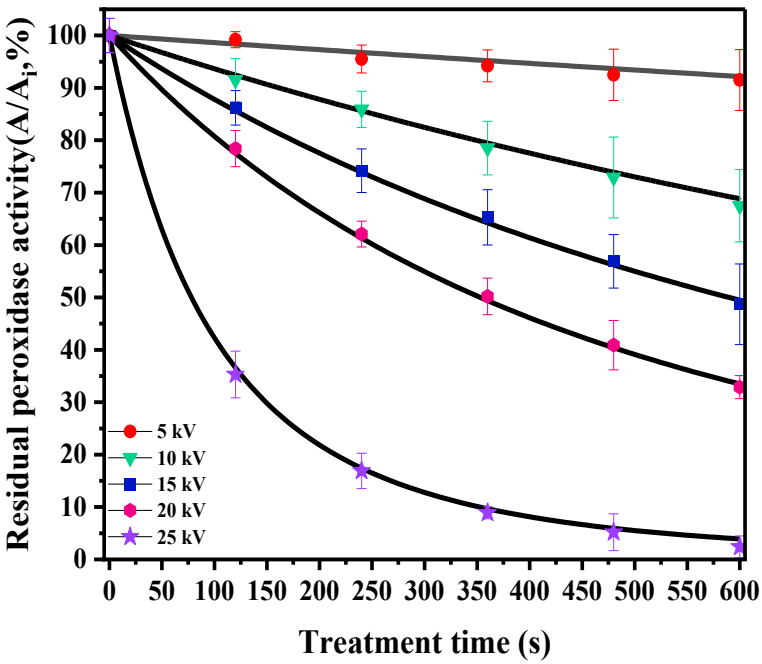
Cold plasma treatment significantly impacted lipase, lipoxygenase, peroxidase, and PPO activity in PMF (Figure 3.1). The freshly milled PMF flour had the lipase, lipoxygenase, peroxidase, and PPO activity of 9.49×10^{-8} kat, 1.70×10^{-7} kat, 3.39×10^{-7} kat, and 7.61×10^{-7} kat, respectively. Cold plasma treatment reduced lipase, lipoxygenase, and peroxidase activities to 5.3 %, 0.8 %, and 5.2 %, respectively, at 25 kV/8 min. PPO enzyme was more vulnerable to cold plasma treatment, with 97.3 % inactivation occurring at 5 kV/6 min; there was no activity of PPO observed in the sample at higher voltages (10-25 kV) of cold plasma treatment. At the lowest intensity of 5 kV/2 min, the activity of lipase, lipoxygenase, peroxidase, and PPO was 96.2 %, 98.7 %, 99.2 %, and 26.9 %, respectively. Increasing the voltage reduces the enzyme activity. For instance, during 4 min in

exposure durations, the lipase activity was 92.1 %, 79.3 %, 66.5 %, 57.2 %, and 16.9 %at 5, 10, 15, 20, and 25 kV, respectively. Similarly, lipoxygenase activity was 96.9 %, 83.1 %, 73.9 %, 18.2 %, and 6.4 %, and peroxidase activity was 95.5 %, 85.9 %, 74.2 %, 62.1 %, and 16.9 %, at 5, 10, 15, 20, and 25 kV, respectively. Similarly, the inactivation extent increases as the exposure time increases. For example, at 20 kV and exposure duration of 2, 4, 6, 8, and 10 min, lipase residual activity is 73.9 %, 57.2 %, 43.2 %, 35.8 %, and 29.5 %, whereas lipoxygenase is 38.3 %, 18.2 %, 9.2 %, 4.9 %, 2.4 %, and peroxidase is 78.4 %, 62.1 %, 50.2 %, 40.9 %, and 32.9 %, respectively. Figures 3.1 (a), (b), (c), and (d) explain the residual enzyme activity of lipase, lipoxygenase, peroxidase, and PPO of PMF after different cold plasma treatment conditions, respectively. The results obtained in this study are in line with the trend reported in the literature. For instance, Gnananethri et al. (2024) found that lipase and lipoxygenase activity in PMF were reduced by 54.1 %and 52.3 %with the cold plasma treatment exposed to 25 kV for 10 min. Wang et al. (2022) discovered that using a dielectric barrier discharge system reduced the lipoxygenase and lipase activity of foxtail millet by 57 and 73 %, respectively, at 25 kV/12 min. Tolouie et al. (2018) observed that cold plasma treatment at 24 kV/ 25 min led to 50 %inactivation in wheat lipoxygenase activity. There are limited studies on the enzyme inactivation of pearl millet, although a few studies have been undertaken on the influence of cold plasma on the enzyme in other food products. Zeng et al. (2023) found that the peroxidase enzyme is completely inactive with dielectric barrier discharge cold plasma in the brown rice powder at 160 kV for 15 min. Chakraborty et al. (2024) found that the 25 kV for 6 min cold plasma treatment resulted in 97.5 and 100 %inactivation of lipase and lipoxygenase activity in whole wheat flour. Surowsky et al. (2013) found that 23 kV for 8.5 min treatment of cold plasma application reduced 12.4 %of polyphenol oxidase enzyme activity. Cold plasma inactivates enzymes by inducing conformational

changes, particularly a reduction in the α-helix content of their structure. This effect arises when enzyme side chains interact with reactive species generated by the plasma, including hydrogen peroxide (H_2O_2), ozone (O_3), and nitrate ions (NO_3^-), as well as singlet oxygen, superoxide (O_2^-), and hydroxyl radicals ($OH^{\cdot}$) (Ganesan et al., 2021; Zargarchi et al., 2024).




Residual lipoxygenase activity (A/A$_i$,%)
Treatment time (s)
5 kV
10 kV
15 kV
20 kV
25 kV


Residual peroxidase activity(A/A$_i$,%)
Treatment time (s)
5 kV
10 kV
15 kV
20 kV
25 kV

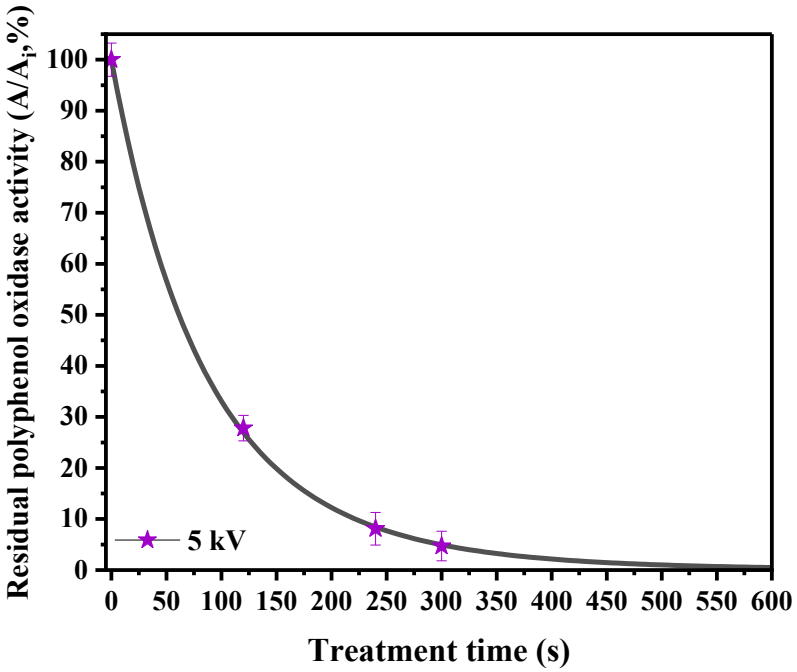


Figure 3.1: The changes in residual enzyme activity of (a) lipase, (b) lipoxygenase, (c) peroxidase, and (d) polyphenol oxidase in pearl millet flour during cold plasma treatment at different treatment conditions.

3.3.3. Influence of steam on the enzyme activity of pearl millet flour

Steaming pearl millet flour at 100 °C deactivates all enzymes, including lipase, lipoxygenase, peroxidase, and PPO. The residual enzyme activity following 1, 2, 3, 4, and 5 min of steam treatment at 100 °C for lipase was 24.9, 17.0, 10.3, 5.2, 2.1 %; for lipoxygenase, it was 18.6, 11.2, 5.8, 1.2, 0 %and for peroxidase it was 34, 21, 15, 7.5, 2.4 %, respectively (Figure 3.2). PPO was the most susceptible enzyme in PMF to steam treatment. The residual PPO activity at 100 °C after 1 min of exposure was 1.3 %. There was no PPO activity observed in PMF when exposed to steam (100 °C) for 2 or more min. Vinutha et al. (2022) discovered that hydrotreatment, hydrothermal, and near-infrared ray combination treatment in pearl millet reduced lipase (47.8 %), lipoxygenase (84.8 %), peroxidase (98.1%), and PPO (100 %). Prashanth et al. (2024) observed that the

hydrothermal treatment (soaking for 3 h/55 °C followed by steaming for 15 min) resulted in a 10 %reduction in lipase activity in pearl millet. Poudel & Rose et al. (2018) discovered that the activities of lipase, lipoxygenase, PPO, and peroxidase lowered up to 81 %, 63 %, 22 %, and 34 %, respectively, as the length of steaming extended to 90 s in the wheat kernel. POD exhibited a greater resistance than lipase, owing to its higher structural rigidity and stronger intermolecular ionic interactions. Guo et al. (2022) discovered that superheated steam treatment of wheat flour (190 °C /10 s) reduced the PPO, lipase, and lipoxygenase activities by 83 %, 100 %, and 99 %, respectively. In steam treatment, the combined effect of moisture and heat enhances enzyme sensitivity, leading to inactivation through uniform heating. This process damages active sites, promotes protein coalescence, and induces aggregation of denatured enzymes. Such aggregation disrupts structural integrity and ultimately abolishes enzyme activity (Chakraborty et al., 2024).

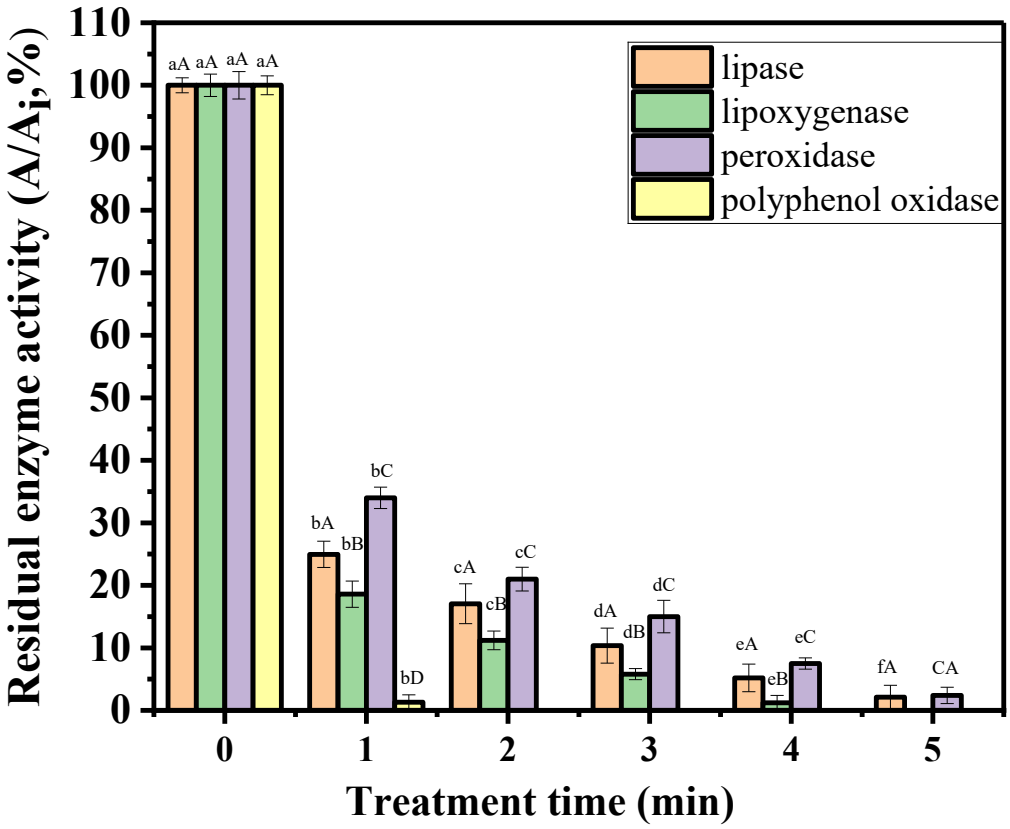


Figure 3.2: The changes in residual enzyme activity of lipase, lipoxygenase, peroxidase, and polyphenol oxidase in pearl millet flour during steam treatment at different periods. The alphabets in small letters (a, b, & c) and capital letters (B, & C) in the superscripts denote that the mean values are statistically different across the columns and rows, respectively, at $p < 0.05$.

3.3.4. Enzymes inactivation kinetics

The inactivation of enzymes in PMF after cold plasma treatment was reported using n^{th} order kinetics. For lipase, linear inactivation kinetics (n = 1), the sum of square errors (SSE) is 1.08, and non-linear inactivation kinetics (n = 1.55), the SSE is 0.67. Conversely, for lipoxygenase, non-linear kinetics (n = 1.15) were best fitted for the cold plasma treatment, having an SSE is 0.45 compared to the linear kinetics SSE is 0.65. Similarly, for peroxidases and polyphenol oxidase

enzymes, the least sum of the square values (n=1.35 and 1.35) are 0.45 and 0.04, than the linear kinetics SSE are 0.56 and 0.10, respectively. The lipase enzyme inactivation rate is 0.026, 0.056, 0.104, 0.149, and 0.636 $U^{n-1} \cdot min^{-1}$ at 5, 10, 15, 20, and 25 kV, respectively. Similarly, lipoxygenase inactivation rate was 0.008, 0.048, 0.078, 0.494, 0.856 $U^{n-1} \cdot min^{-1}$ at 5, 10, 15, 20, and 25 kV, respectively (Table 3.2). Similarly, peroxidase has the 0.008, 0.039, 0.079, 0.133, 0.602 $U^{n-1} \cdot min^{-1}$ inactivation rates, respectively. PPO showed the highest inactivation rate compared to the remaining enzymes. For instance, its inactivation rate is 0.7015 $U^{n-1} \cdot min^{-1}$ at 5 kV. The greater the extent of inactivation, the more sensitive the enzyme is to treatment (Chakraborty et al., 2024). Kinetic modeling indicates that polyphenol oxidase is the most sensitive to cold plasma, while peroxidase is the most resistant, with a sensitivity to voltage (S_v) value of 1.660.

Table 3.2: The inactivation rate of enzymes in pearl millet flour during atmospheric cold plasma treatments.

V_{rms} (kV)	*k* value (× 10^{-2}, min^{-1})			
	Lipase	Lipoxygenase	Peroxidase	Polyphenol oxidase
5	2.6 ± 0.4^{aB}	0.8 ± 0.6^{aC}	0.8 ± 0.5^{aC}	70.1 ± 0.6^{aD}
10	5.6 ± 0.9^{bB}	4.8 ± 0.4^{bC}	3.9 ± 0.9^{bA}	-
15	10.4 ± 0.5^{cB}	7.8 ± 0.9^{cC}	7.9 ± 1.1^{cC}	-
20	14.9 ± 1.1^{dB}	49.4 ± 1.5^{dC}	13.3 ± 0.4^{dA}	-
25	63.6 ± 0.9^{eB}	85.6 ± 1.1^{eC}	60.2 ± 0.3^{eA}	-

The alphabets in small letters (a, b, & c) and capital letters (B, & C) in the superscripts denote that the mean values are statistically different across the columns and rows, respectively, at $p < 0.05$

3.3.5. Influence of cold plasma and steam treatments on the bioactives and antinutrients of pearl millet flour

Based on the kinetic modeling, the optimized condition for achieving over 90 %enzyme inactivation of all enzymes was identified as steam treatment at 100 ºC/4 min and cold plasma treatment at 25 kV/8 min. Table 3.3 shows the bioactive and antinutrient properties of the untreated, steam (100 ºC/4 min), and cold plasma-treated (25 kV/8 min) PMF. There were

significant changes in the bioactives and antinutrients in PMF after cold plasma and steam treatments. Total phenolic content (TPC) was reduced from 343 to 154 mg GAE·kg^{-1} in the steam-treated sample and 152 mg GAE·kg^{-1} in the cold plasma-treated sample. Total flavonoid content (TFC) was reduced from 58.9 to 48.28 mg CE.kg^{-1} in the steam-treated sample. It was reduced to 49.72 mg CE·kg^{-1} in the cold plasma-treated sample. Charu et al. (2024) discovered that cold plasma treatment reduced TFC by 10 g/hg and TPC by 2 g/hg in pearl millet at 2 kV/5 min. Sarkar et al. (2023) reported a similar result in cold plasma-treated PMF. After 20 min of cold plasma exposure at 30 kV, TPC decreased by 16.3 g/hg and TFC decreased by 33.3 g/hg. Steam treatment degraded the TPC and TFC by thermal hydrolysis and oxidation (Zhang et al., 2019). Plasma discharge produces high-energy electrons that directly interact with phenolic compounds, causing their breakdown. Through oxidative degradation, aromatic structures are converted into hydroxylated derivatives and quinone compounds. Furthermore, flavonoids are oxidatively degraded by reactive oxygen species (ROS) produced throughout the treatment, leading to a reduction of TFC (Misra et al., 2014).

Phytic acid content was reduced from 363 to 292 mg·kg^{-1}in steam treatment, and 275 mg·kg^{-1} in cold plasma treatment. Tannin content was reduced from 418 mg GAE·kg^{-1} to 347 mg GAE·kg^{-1} in the steam-treated sample and 93 mg GAE·kg^{-1} in the cold plasma-treated sample. Charu et al. (2024) reported that cold plasma treatment of PMF at 2 kV for 5 min reduced tannin and phytic acid levels by 18 g/hg and 57 g/hg, respectively. This reduction is attributed to ROS, which cleave chemical bonds and disrupts the phytate ring structure, thereby lowering tannin and phytate concentrations (Sadhu et al., 2017). According to Sarkar et al. (2023), cold plasma exposure decreased anti-nutritional components such as tannins (0.98 to 0.81 g/hg) and phytates (9.25 to 6.71 g/hg) at 30 kV for 20 min. Sade (2009) discovered that roasting reduced tannins (0.51 to 0.29

mg/100 g) and phytate (0.21 to 0.11 mg/100 g) in pearl millet. Gwekwe et al. (2024) found that roasting pearl millet considerably lowered the phytate concentration from 0.66 to 0.41 g/hg and the tannin amount from 402 to 321 mg/g. Prashanth et al. (2024) analyzed the influence of hydrothermal processing on the antinutritional characteristics of pearl millet. Treatment with soaking temperatures (55 °C), soaking lengths (3 h), and steaming times (15 min) reduced tannins by 14.3 g/hg and phytates by 3.82 g/hg, respectively. The reduction of tannins and phytates can be attributed to their heat-sensitive nature, which promotes breakdown at elevated temperatures. In cold plasma treatment, reactive oxygen and nitrogen species interact with the functional groups of these antinutritional factors, leading to their degradation (Sarkar et al., 2023).

The antioxidant activity (AOA) of PMF after the steam and cold plasma treatment did not change significantly. Wang et al. (2022) discovered no significant change in the DPPH radical scavenging capacity of foxtail millet following treatment with the dielectric barrier discharge-air cold plasma. Tolouie et al. (2018) also found the same trend in the antioxidant capacity of results by cold plasma-treated wheat germ. However, Sarkar et al. (2023) found the opposite trend. They reported a significant reduction in AOA in the cold plasma-treated PMF. These contrasting findings may reflect differences in plasma conditions (e.g., voltage, duration, gas composition) or variations in the grain matrix and phenolic composition. ROS and RNS generated during plasma can either liberate bound antioxidants, maintaining activity, or oxidize phenolic compounds, leading to loss of functionality. Further research on free vs. bound phenolics in PMF is needed to clarify these responses.

Table 3.3: Effect of cold plasma and steam treatments on antinutrient and bioactive compounds of pearl millet flour

parameter	Cold plasma treatment (25 kV/8 min)	Steam treatment (100 °C/4 min)	Untreated
Total flavonoids (mg CE.kg^{-1})	49.7 ± 27.2^{a}	48.2 ± 21.2^{a}	58.9 ± 12.5^{b}
Total phenolics (mg GAE.kg^{-1})	152.9±25.3^{a}	154.8 ±30.0^{a}	343.7 ±25.7^{b}
Antioxidant activity (mg GAE.kg^{-1})	99.1 ± 0.0^{a}	98.9 ± 0.0^{a}	98.3 ±0.0^{a}
Phytic acid (mg.kg^{-1})	275.8 ± 1.1^{a}	292.2 ± 32.5^{a}	363.2± 49.5^{b}
Tannin content (mg GAE .kg^{-1})	93.0 ±9.3^{a}	347.5 ± 6.6^{b}	418.4± 6.8^{c}

The dissimilar alphabets (a, b & c) in the superscripts denote that the mean values are statistically different across the treatment conditions at $p < 0.05$.

3.3.6. Influence of cold plasma and steam treatments on the lipid oxidation products of pearl millet flour

Free fatty acid (FFA) value determines the extent of hydrolytic rancidity by quantifying the amount of free fatty acids released. Free fatty acid values for the untreated, cold plasma, and steam-treated PMF were 137.31, 93.24, and 96.23 mg NaOH/100g (Table 3.4). The TBARS test measures the extent of formation of the secondary lipid oxidation products in the PMF. TABRS values for the untreated, cold plasma, and steam-treated PMF were 4.00, 2.57, and 2.73 mg MDA/kg, respectively. The extent of hydrolytic rancidity and secondary lipid oxidation was reduced in both steam and cold plasma-treated samples than the untreated flour. During the cold plasma and steam treatment, the generated reactive species and a heat, moisture combination inactivate the lipase and lipoxygenase enzymes, which are responsible for the FFA production and secondary lipid oxidation, leading to the reduction in the lipid oxidation products in the PMF. At the same time, there was a greater reduction of hydrolytic rancidity in the cold plasma-treated sample than in the steam-treated sample. Reactive species generated during cold plasma treatment chemically modify and alter the enzyme-substrate interaction (Misra et al., 2016). It oxidizes and breaks down the unsaturated fatty acids directly, leading to a reduction in the FFA. In the steam treatment, it primarily inactivates the enzyme through heat but is less effective in modifying the lipid structure

(Gao et al., 2023 & Bahrami et al., 2015). Gnananethri et al. (2024) found that FFA content (0.842-9.112%) was lowered after cold plasma treatment (25kV/10min) of the pearl millet flour, compared to untreated flour. Bhati et al. (2016) observed that dry heat treatment (120min) reduced the FFA Content (445.6-330.2 mg/kg) in pearl millet flour. Peroxide value indicated the extent of the peroxides and hydroperoxides formed during the lipid oxidation. Peroxide value for the untreated, cold plasma, and steam-treated PMF were statistically the same (around 3.0 meq O_2/kg). There is no significant difference ($p > 0.05$) in peroxide value between the treated and untreated pearl millet flour. Narayanan & Ravikumar (2017) found that the peroxide value remained close to zero till 45 days of storage of PMF through roasting. Nantanga et al. (2008) observed that the peroxide value of the untreated and wet thermally treated fresh pearl millet flour remains the same. Peroxide value will be increased due to the increase in moisture, hydrolytic, and oxidative rancidity, causing enzymes (Gnananethri et al., 2024). Peroxides are the end products in the rancid reactions, at the initial days, under good storage conditions. Oxidation reaction takes some time to initiate and propagate the measurable peroxide accumulation (Oh et al., 2023). In the cold plasma and steam treated flour, inactivation of enzymes suppresses the rancid mechanism leading to the formation of the peroxides in the flour. A storage study is required to measure the changes in the peroxide value.

Table 3.4: Effect of cold plasma and steam treatments on functional properties and lipid oxidation products of pearl millet flour

parameter	Cold plasma treatment (25 kV/8 min)	Steam treatment (100 °C/4 min)	Untreated
Water absorption capacity (g/g)	2.20 ± 0.4^{a}	1.55 ± 0.1^{b}	1.56 ± 0.1^{b}
Oil absorption capacity (g/g)	1.94 ± 0.2^{a}	1.47 ± 0.08^{b}	1.43 ± 0.1^{b}
Emulsification capacity (g/hg)	54 ± 0.05^{a}	50 ± 0.1^{b}	50 ± 0.09^{b}
FFA value (mg NaOH/100g)	93.24 ± 1.2^{a}	96.23 ± 0.9^{b}	137.31 ± 1.1^{c}
Peroxide value (meq O_2/kg)	3.02 ± 0.01^{a}	3.01 ± 0.01^{a}	3.01 ± 0.02^{a}
TBARS (mg MDA/kg)	2.57 ± 0.1^{a}	2.73 ± 0.3^{a}	4.00 ± 0.1^{b}

The dissimilar alphabets (a, b & c) in the superscripts denote that the mean values are statistically different across the treatment conditions at $p < 0.05$.
FFA, free fatty acids: TBARS, thiobarbituric acid reactive substance

3.3.7. Influence of cold plasma and steam treatments on the protein structure of pearl millet flour

Secondary structure of the pearl millet protein consists of the α-helices, β-sheets, β-turns, and random coils in the ranges between 1600 and 1700 cm^{-1} in the FTIR spectra. The α-helix (1650-1660 cm^{-1}), β-sheet (1620-1640,1680 cm^{-1}), β-turn (1660-1680,1694 cm^{-1}), and random coils (1640-1650 cm^{-1}) were identified in the protein amide I region (Pulivarthi et al., 2025). Pearl millet flour has 20.8 g/hg α-helix, 34.0 g/hg β-sheet, 13.8 g/hg β-turn, and 29.1 g/hg random coil (Table 3.5). After cold plasma treatment (25 kV/8 min), the α-helix, β-sheet, β-turn, and random coil portions are 8.3 g/hg, 36.8 g/hg,11.5 g/hg, and 31.4 g/hg, respectively. The proportions in secondary structure after steam treatment (100 ºC/4 min) are 16.3 g/hg, 20.3 g/hg, 8.7 g/hg, and 37.0 g/hg for the α-helix, β-sheet, β-turn, and random coil portions, respectively. Both cold plasma and steam treatment have a significant effect on the secondary structure of the pearl millet flour. With cold plasma treatment, slight modifications in protein secondary structure are due to the unfolding and oxidation of amino acid side chains through ROS and RNS. These reactive species

break the intermolecular and intramolecular hydrogen bonds, resulting in a reduction in the α-helix and β-turn regions, and a relatively slight increase in the β-sheet and random coil regions (Li et al., 2024). Sarkar et al. (2023) found that cold plasma treatment (20-30 kV/10-20 min) of the PMF leads to a significant reduction in β-sheet and an increase in the α-helix structure. Misra et al. (2015) observed that the higher voltage condition (60 kV/5min) of the cold plasma treatment leads to an increase in the β-sheet +anti-parallel β-sheet and a reduction in the α-helix in the wheat flour. The structural changes in the protein depend on the voltage intensity and exposure duration. Steam treatment has a greater effect on the secondary structure of proteins than cold plasma treatment. Ma et al. (2021) found that heating wheat flour led to the transformation of α-helices and β-turns into β-sheets and random coils. Baltacioglu et al. (2015) found that increased temperature led to the aggregation of random coil and β-sheet in mushroom polyphenol oxidase. Heat and moisture induction led to protein restructuring through the disruption of the hydrogen bonds and hydrophobic interactions (Vinutha et al., 2022).

Table 3.5: Effect of cold plasma and steam treatments on secondary structure of protein in pearl millet flour

Secondary structures	Cold plasma treatment (25 kV/8 min)	Steam treatment (100 °C/4 min)	Untreated
α - helix (g/hg)	18.3 ± 2.3^{a}	16.3 ± 2.7^{a}	20.8 ± 3.1^{b}
β - sheet (g/hg)	36.8 ± 4.1^{a}	20.3 ± 3.8^{b}	34.0 ± 4.9^{a}
β - turn (g/hg)	11.5 ± 2.1^{ab}	8.7 ± 2.5^{b}	13.8 ± 1.9^{a}
Random coil (g/hg)	31.4 ± 2.3^{a}	37.0 ± 3.4^{b}	29.1 ± 2.5^{a}

The dissimilar alphabets (a, b & c) in the superscripts denote that the mean values are statistically different across the treatment conditions at $p < 0.05$.

3.3.8. Influence of cold plasma and steam treatments on the functional properties of pearl millet flour

Cold plasma treatment has a significant effect on the functional properties of pearl millet flour, surpassing that of steam treatment, such as oil absorption capacity, water absorption capacity, and emulsification capacity. Pearl millet flour has 1.56 g/g, 1.43 g/g, 50 g/hg of the water absorption capacity, oil absorption capacity, and emulsification capacity, respectively. Cold plasma-treated flour has a water absorption capacity of 2.2 g/g, an oil absorption capacity of 1.94 g/g, and an emulsification capacity of 54 g/hg. After steam treatment, the water absorption capacity, oil absorption capacity, and emulsification capacity of pearl millet flour are 1.55 g/g, 1.47 g/g, and 50 g/hg, respectively. There was no significant ($p > 0.05$) change in the functional properties of the steam-treated sample with respect to the untreated flour. All the functional properties were enhanced with cold plasma treatment. Sarkar et al. (2023) and Lokeswari et al. (2021) observed a similar trend in increasing the oil and water absorption capacities, as well as the emulsifying capacity, through cold plasma treatment of pearl millet flour. The increase in water absorption may be due to the ions and radicals generated during plasma treatment, which enhances the hydrophilicity of the flour. Plasma induces the depolymerization and partial oxidation of starch, resulting in the production of carboxylic starch. The increase in water absorption is desirable and helps to maintain the consistency in baking applications. The increase in surface hydrophobicity of the protein groups is responsible for enhancing the emulsification capacity. Changes in the oil absorption capacity of the cold plasma-treated flour might be due to the inclusion of non-polar amino acids and protein structures (Sarkar et al., 2023; Lokeswari et al., 2021).

3.3.9. Influence of cold plasma and steam treatments on the physicochemical properties of pearl millet flour

The PMF had bulk, true, and tapped density values of 850,1370, and 740 kg/$m^{3,}$ respectively (Table 3.6). There was no significant ($p>0.05$) variation in the density of the steam and cold plasma-treated samples. Lokeswari et al. (2021) did not notice a significant difference in the loose, tap, and bulk density of cold plasma-treated pearl millet flour. The geometric mean diameter of pearl millet flour was in the range of 389 to 392 μm. Cold plasma exposure did not alter the flour particle size because the treatment acts mainly on surface chemistry through reactive species, without applying mechanical disruption or significant heat stress that could fragment or aggregate particles (Chauhan et al., 2023). Cold plasma and steam treatment did not affect any color profile of the pearl millet (*L**=50.75*, a**=9.67*, b**=24.49*)*. But cold plasma-treated PMF showed lesser water activity and moisture content compared to the steam-treated and untreated PMF. For instance, cold plasma-treated PMF had 7.1 % moisture content with 0.36 water activity, whereas untreated and steam-treated PMF had 0.48 water activity and moisture content (7.3 g/hg). The moisture content is stable in steam treatment due to reaching the equilibrium moisture content, followed by the initial condensation and dehydration phase after the treatment (Jin et al., 2021). Charu et al. (2024) discovered that PMF moisture content decreased from 5.65 g/hg to 4.17 g/hg after cold plasma treatment. Sarkar et al. (2023) also discovered a small fall in moisture content from 10.75 g/hg to 10.02 g/hg (db) following cold plasma exposure at 30 kV/20 min. The breakdown of water molecules into oxygen radicals could account for the decrease in moisture levels. Furthermore, it is probable due to water molecules absorbed by starch granules and dissociated, hastening the association (Wongsagonsup et al. 2014). Steam and cold plasma treatments did not alter the protein, ash, or damaged starch content of pearl millet flour ($p > 0.05$). Similar results are discussed

by Charu et al. (2024). They did not see any change in the ash and protein content of the PMF. This stability can be attributed to the fact that plasma primarily modifies surface chemistry without affecting bulk nutrient composition, while steam treatment under controlled conditions does not degrade proteins, volatilize minerals, or mechanically disrupt starch granules (Ganesan et al., 2021; Guo et al., 2020).

Table 3.6: Effect of cold plasma and steam treatments on physical and compositional attributes of pearl millet flour

Attributes	Cold plasma treatment (25 kV/8 min)	Steam treatment (100 °C/4 min)	Untreated
Bulk density (kg/m³)	850 ± 0[a]	852 ± 0[a]	849 ± 0[a]
True density (kg/m^3)	1370 ± 0.0[a]	1360 ± 0.0[a]	1370 ± 0.0[a]
Tapped density (kg/m³)	730 ± 0[a]	730 ± 0[a]	730 ± 0[a]
Water activity	0.36 ± 0.03[a]	0.48 ± 0.05[b]	0.48 ± 0.02[b]
Damaged starch (%)	9.80 ±0.00[a]	10.39 ± 0.07[b]	10.25 ± 0.11[b]
Moisture (%)	7.1 ± 0.1[a]	7.3 ± 0.1[b]	7.3 ± 0.1[b]
Protein (%)	10.5 ± 0.3[a]	10.5 ± 0.2[a]	10.5 ± 0.1[a]
Ash (%)	2.4 ± 0.2[a]	2.3 ± 0.0[a]	2.3 ± 0.1[a]
d_{gw} (µm)	389.0 ± 2.7[a]	392.0 ± 3.2[a]	387.0 ± 2.9[a]
Color profile			
*L**	50.7 ± 0.2[a]	50.9 ± 0.3[a]	50.7 ± 0.4[a]
*a**	9.67 ± 0.1[a]	9.6 ± 0.4[a]	9.7 ± 0.2[a]
*b**	24.4 ± 0.2[a]	23.9 ± 0.3[a]	24.1 ± 0.3[a]

The dissimilar alphabets (a & b) in the superscripts denote that the mean values are statistically different across the treatment conditions at $p < 0.05$.

3.4. Conclusion

Steam treatment at 100ºC/4 min and cold plasma treatment at 25 kV/8 min inactivated all the enzymes (peroxidase, lipase, polyphenol oxidase, and lipoxygenase) in pearl millet flour more than 95 %. The peroxidase enzyme shows more resistance to the cold plasma and steam treatment. All the enzymes, such as lipase, lipoxygenase, peroxidase, and polyphenol oxidase, deviate from the first–order kinetics. Polyphenol oxidase is the most sensitive enzyme to steam and cold plasma treatment. Peroxidase is the most resistant enzyme to the treatment, it needs to be considered in the process design in terms of improving the quality. A higher reduction of the tannin (77.7 g/hg) and phytic acid (24 g/hg) content was seen in the cold plasma treatment samples than in the steam treatment. The physicochemical characteristics of the pearl millet flour are not affected by the cold plasma treatment, and it helps to preserve the heat-sensitive nutrients. Both treatments reduce the formation of the secondary lipid oxidation products, such as the free fatty acids and TABRS. Cold plasma treatment enhances the functional properties such as the water absorption capacity (1.56-2.20 g/g), oil absorption capacity (1.43-1.94 g/g), and emulsification capacity (50-54 g/hg). The study will extend its focus on assessing the shelf life and impact on the nutrients in future research. Future studies should evaluate the sensory attributes of end-use products formulated with the treated flour to fully understand the practical impact of these processing-induced changes.

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Chapter 4

Cold Plasma Treatment as an Intervention Strategy to Achieve Safety in Pearl Millet Flour

Abstract

Pearl millet flour (PMF) is called the nutri-cereal due to its rich nutritional profile, with omega-3 fatty acids and essential amino acids. However, PMF is contaminated with pathogenic species due to poor and unhygienic postharvest practices. This study aims to inactivate Salmonella spp, which cause foodborne illnesses, using the cold plasma treatment (20-28 kV/20-60 min). The effectiveness of the treatment is observed through different combinations of treatments, such as the contaminated pearl millet grains are treated with cold plasma treatment (A), grains tempered with plasma-activated water (B), and the contaminated pearl millet grain is milled to flour and treated with cold plasma treatment (C). Furthermore, with cold plasma treatment as all possible combinations of treatment such as A+B, B+C, A+C, and A+B+C. Cold plasma induced the highest microbial inactivation of 3.0 log cycle, with the combination treatment of A+B+C such as the cold plasma treatment of the grain, followed by tempering with plasma-activated water and cold plasma treatment of the flour at 28 kV/60 min. Through the individual treatment of the grains (A) and the combination of A + C, the highest reduction of 1.4 and 2.6 log CFU/g at 28 kV/60 min, respectively was achieved. Except for the condition 28 kV/60 min for the A+ B + C combination, cold plasma treatment at all treatment conditions did not affect the bulk, true, tapped density, geometric mean diameter, protein, and ash content of the PMF, but it reduced the water activity and moisture content of the flour. A+B+C treatment condition shows a decrease in true, bulk, tapped density, an increase in the geometric mean diameter, and changes in the color profile observed due to the tempering of the grains. Cold plasma treatment inactivates the pathogenic species without compromising the PMF quality attributes.

Keywords: Pearl millet, *Salmonella*, Cold plasma treatment, Plasma-activated waters

4.1. Introduction

The Food and Agriculture Organization (FAO) declared 2023 as the International Year of Millets (IYM 2023) to emphasize the importance of millets (Nutri-Cereals) as a sustainable food source (FAO, 2023). Climate change and socioeconomic repercussions are predicted to significantly threaten agriculture and food security in the 21st century. Millets have the resilience to adverse climatic conditions with nutritional benefits (Kumar et al., 2025). Pearl millet (*Pennisetum glaucum*), one of the millet crops, thrives in harsh, arid environmental conditions, making it a climate-resilient crop (Aher et al., 2025). It is called as the energy-rich cereal, having the beneficial unsaturated fats, including omega-3s, and a robust nutritional profile of 2.7-7.1% fat, 8.5-15.1% protein, 58.5-72.5% carbohydrates. This gluten-free grain with high fiber content aids in regulating blood sugar and promoting heart health. The essential and non-essential amino acids with leucine (1.70%), phenylalanine (0.71%), threonine (0.52%), isoleucine (0.58%), glutamic acid (3.58%), aspartic acid (1.32%), proline (1.07%), and alanine (1.33%) enhance the total amino acid profile of the pearl millet (Hemanth et al., 2025).

According to Future Market Insights (2025), pearl millet flour (PMF) is started being commercialized on a large scale, increasing the global millet flour market to about USD 5.4 billion with an increase of 5.9% in 2025. This nutrient-enriched PMF is frequently contaminated and exhibits poor microbiological quality (Naraharasetti et al., 2025a). Pathogenic species such as *Staphylococcus aureus* oscillated between 3.32 and 5.67 log CFU/g. The microbial load of *Bacillus cereus* varied from 7.18 to 10.25 log CFU/g. microbial population of *Escherichia coli* varied from 2.13 to 6.08 log CFU/g. For fungal flora, the loads varied from 2.84 to 8.25 log CFU/g. Enterobacteria, the loads oscillated from 3.02 to 3.50 log CFU/g in the pearl millet flour originating

from poor hygiene and post-harvest practices, leading to flour contamination (Christelle et al., 2021). Contaminated flour will lead to food intoxication and food poisoning (Kesby et al., 2024). Thus, effective treatment is essential for maintaining the quality and safety of PMF. Some existing treatment methods are effective in achieving microbial safety of PMF. Choudhary et al. (2025) studied the effect of hot water blanching at 98 °C/30 s on pearl millet flour. It leads to a reduction of 4.45, 4.14, and 4.47 log cycle for aerobic mesophiles, yeast, and coliforms, respectively. Steaming at 100 °C for 20 min leads to a reduction of coliforms and aerobic mesophiles of 5.38 and 4.71 log cycles, respectively. Narayanan and Ravikumar (2017) found that roasting (180 °C/30 s) of pearl millet grains has reduced aerobic mesophiles by 4.69 log CFU/g. Dikkala et al. (2018) studied heat treatment with a rotary dryer at 150-170 °C/1.5 min, which reduced the 0.28 log CFU/g fungal count in PMF. Choudhary et al. (2025) studied the significance of ultrasound treatment on pearl millet safety. They got a reduction of 4.83, 4.61, and 4.53 log CFU/g aerobic mesophilic, yeast, and coliforms count, respectively.

Salmonella is the key target microorganism in the flour-based products that raises concerns nowadays during outbreaks and has an impact on public health, causing foodborne illness. Salmonella outbreak leads to 12 illnesses, with 3 being hospitalized after consuming the contaminated wheat flour (CDC, 2023). Microbial contamination in grains was reported to be in the outer layer. This results in grain germ and bran having higher quantities of microbial contamination (Rivera et al., 2022). Tempering is a pre-milling step in the wheat milling industry, where in the grains are hydrated, followed by a conditioning step that lasts 24 hours. This operation helps toughen the bran and soften the endosperm, which boosts flour extraction rates and quality. Commercial milling operations mostly use water for the tempering step. Recent evidence shows that bacteria might be capable of adapting to temperatures and pH levels that are not ideal, which

could ultimately lengthen the time it takes to kill them (Doddabematti et al., 2024). There is a need for novel and energy-efficient technology to inactivate the microorganism that is adapting to the existing methods.

Cold plasma is a novel non-thermal technology that generates reactive radical species containing the excited ions, atoms, and electrons that interact with the food surface, leading to microbial activation in grain-based products (Chakraborty et al., 2024). Plasma-activated water (PAW) is produced by exposing the water to atmospheric cold plasma, which has high reactivity through reactive species and antimicrobial properties (Rivera et al., 2024). Charu et al. (2024) found that cold plasma treatment of pearl millet achieved 5.83 and 4.48 log reduction of the aerophilic mesophilic, and total yeast count at 2 kV/5 min. They found 3.47 and 3.6 log reduction of the *E. coli* and *Enterococcus spp*. Los et al. (2018) found that cold plasma treatment (80 kV) on the wheat leads to 1.5 and 2.5 log cycle reduction of the bacteria and fungi within 20 min. Park et al. (2020) found that brown rice achieved more than 1.5 log reduction of the bacteria, yeast, and molds after the 20 kV/10 min of cold plasma treatment. To date, no study has been conducted on the effect of combining cold plasma treatment and plasma-activated water tempering (PW) on the inactivation of Salmonella to achieve microbial safety in pearl millet flour. The present study evaluates the efficacy of the cold plasma treatment and PW tempering in inactivating *Salmonella* in pearl millet grain and flour. Furthermore, this study evaluates the effect of cold plasma treatment on the physicochemical properties, such as the moisture content, protein, fat, density, and particle size of the pearl millet flour.

4.2. Materials and Methods

4.2.1. Materials

Pearl millet grain was used for the current study, purchased from the Ancient Wisdom Organic Bajra Grain, Mumbai, Maharashtra, India. Pearl millet grain is milled with lab scale (Greatrue high speed multifunction grinder type: HC-150, 1500 W, 110V). The *Salmonella* (Newport—ATCC 6962, Typhimurium ATCC 14802, Kentucky—ATCC 9263, and Enterica— ATCC 8326) were procured from the Feed Toxicology and Microbiology Laboratory, Kansas State University. Bacterial stock cultures are stored at -80 ℃ in tryptic soy broth supplemented with 30% glycerol until further use. All the media and other chemicals were purchased from Fisher Scientific, unless mentioned otherwise.

4.2.2. Experimental design

The current study employed with full factorial design (3^2). The two independent variables are RMS voltage (20-28kV) and time (20-60 min). Figure 4.1 shows the overview of the experimental procedure of study. *Salmonella* inoculated grains are initially treated with cold plasma treatment at room temperature under different voltages (20, 25, 28 kV) and time periods (20, 40, 60 min). Following the grains are tempered (moisture content 16 %) with the plasma-activated water, which is done at similar treatment conditions of the grains. Moisture content of the pearl millet grains is determined by the hot air oven techniques (AACC Method 44-40.01). The amount of tempering solution is calculated based on the initial moisture content (11%). The following grains are agitated mechanically in the tempered bins. The tempering of the grain was done for 24 hours for uniform distribution of moisture inside the grain. The grains are milled into flour. Then the flour was subjected to cold plasma treatment at the same voltage and time duration. A multipin-plane plasma reactor (Model: LCP-MP, Ingenium Technologies, Odisha, India) operating in batch mode in atmospheric air conditions was utilized for the entire study. PAW was generated by exposing the distilled water in the petriplates under the plasma-generated pins. Due to the large surface area of

the petriplates, plasma-generated radicals dispersed into the water more uniformly and effectively. The concentration and intensity of the radicals depend on the applied voltage and time duration. The distance (4 cm) between the plasma-generated pins and the petriplate is constant throughout the experiment. During the combined treatment conditions, such as A+B, B+C, A+C, and A+B+C, all steps are exposed under the cold plasma with the same voltage and time duration.The reduction in microbial population was counted at every step after the cold plasma treatment. All the possible conditions of experiments in each step with cold plasma treatment were conducted.

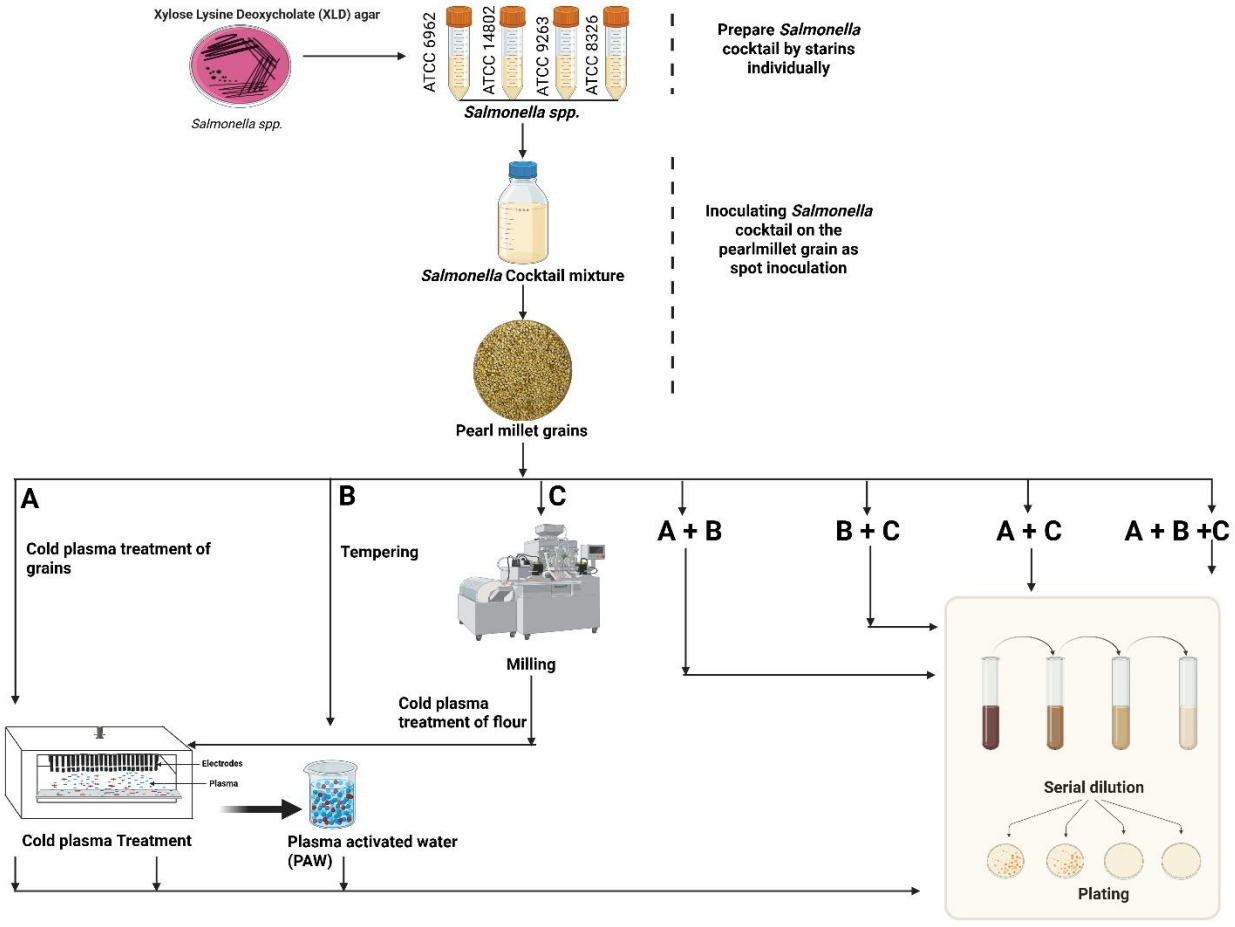

Figure 4.1: Schematic view of the experimental plan of the study, A, cold plasma treatment on the grains (25-28 kV/20-60 min); B, Tempering with plasma-activated water (25-28 kV/20-60 min); C, cold plasma treatment on the flour (25-28 kV/20-60 min).

4.2.3. Inoculum preparation

Defrosted cultures (100 µL) of *Salmonella* were inoculated and incubated twice in individual 10 mL tryptic soy broth (TSB). The inoculated culture was streak-plated in Tryptic Soy Agar (TSA). A single, well-isolated colony of each *Salmonella* from a TSA plate was inoculated into individual TSB (10 mL) media tubes and incubated. Inoculated culture (100 µL) was spread plated on the TSA plates. After incubation, the culture is extracted from the TSA plates with the peptone water (0.1%) by scraping the media. An equal volume of the prepared cell suspension was mixed to make the strain inoculum cocktail for the experiments. *Salmonella* incubation was conducted at 24 h at 37 ℃. Salmonella has an inoculum concentration (plated in TSA) obtained through this process has 1×10^{12} colony-forming unit/gram (CFU/g). An equal amount of cell suspension was combined with the media and broth in every step to maintain the standard concentration.

4.2.4. Application of inoculum, cold plasma treatment, and tempering procedure

25 g of pearl millet grains are inoculated with the spot inoculation procedure (1mL) in re-sealable bags. The kernels were agitated mechanically in the shaker to evenly distribute the inoculum after inoculation. Pearl millet grains have achieved the inoculation level of 9.0 log CFU/g for *Salmonella* when inoculated grains rested for 24 hours at room temperature (20-24℃).

4.2.5. Enumeration of Salmonella

After treatment, the sampled pearl millet flour and grain (10 g) were blended with 90 mL of sterile buffered peptone water using a stomacher (Seward) for 2 min at 300 rpm. The blended samples were then serially diluted in 0.1% sterile buffered peptone water, and appropriate dilutions were spread-plated (in duplicates each time, 100 µL) in xylose lysine deoxycholate (XLD) agar, overlayed with TSA. The inoculated plates were then incubated at 37°C for 24 h. Colonies in the XLD plates, the black colonies are counted as Salmonella. Enumerated colonies expressed as log

$_{10}$ CFU/g, and the reduction was estimated as the difference between the microbial count of treated and untreated cold plasma treatment.

4.2.6. Physicochemical analysis

Analyzing the proximate composition of the flour, moisture content was measured with hot air oven techniques (AACC Method 44-40.01), protein content was measured with the combustion method (AACC Method 46-30.01), and ash content using a muffle furnace (AACC Method 08-01.01). The color of the four was measured using the MiniScan EZ 4000 Portable Spectrophotometer (Hunter Lab). Particle size analysis was conducted using a Ro-Tap Sieve shaker (Hosokawa Micron, E20). Water activity was measured using Aqua Lab (AquaLab 4 TE, Decagon Devices). Bulk density was measured using the Winchester cup arrangement (Seedburo Equipment Co., Des Plaines, IL, USA). True density was measured using the gas pycnometer (Model: Quantachrome Ultrapyc 1200e, Odelzhausen, Germany), and tapped density was measured using the Autotap Density Analyzer (Quantachrome Instruments, Boynton Beach, FL, USA). The bulk, true, tapped density, and geometric mean diameter were measured using the method by Pulivarthi et al. (2022).

4.2.7. Statistical analysis

Three replications were done for all the experiments in this study. The results are expressed as mean ± SD. The Origin Pro 2023b software (Origin Lab Corporation, Northampton, MA, USA) was used for the analysis of variance (ANOVA).

4.3. Results and Discussion

4.3.1. *Salmonella* inactivation during plasma-activated water (PW) tempering, cold plasma treatment on grain and flour

Figure 4.2 (a), (b), and (c) explain the *Salmonella* inactivation achieved through cold plasma treatment of the grains (A), flour (C), and PW tempering (B) of pearl millet under various voltages (20-28 kV) and time durations (20-60 min).

4.3.1.1. Inactivation during plasma treatment on grain (A)

Cold plasma treatment on the grain (A) achieved the log reduction of 0.18, 0.26, 0.31, 0.42, 0.60, 1.01, 0.85, 1.39, 1.40 log CFU/g at 20 kV/20 min, 20 kV/40 min, 20 kV/60 min, 25 kV/20 min, 25 kV/40 min, 25 kV/60 min, 28 kV/20 min, 28 kV/40 min, 28 kV/60 min, respectively. At 20 kV, lower time exposure did not show a significant reduction in the *Salmonella* population; 28 kV treatment condition shows a significant log reduction. Voltage has a greater effect on the microbial reduction than the exposure time. Few studies explored the efficacy of cold plasma technology in disinfecting *Salmonella* in wheat grains. Thomas-Popo et al. (2019) achieved a 2.7 log reduction of *S. enterica* with cold plasma treatment (44 kV/20 min) in the wheat grains. Niemira et al. (2012) got 1.16 log reduction of *Salmonella* (*S. Anatum F4317*) through cold plasma treatment on the almonds. Casado et al. (2024) worked on the efficiency of the plasma-processed air, which leads to a 2.1 log reduction of *Enterococcus faecium* NRRL B-2354, which is a suitable control indicator as resistant as *Salmonella i*n the wheat grain. The (RNS) reactive nitrogen species, such as nitrite (NO^-_2), nitrate (NO^-_3), nitric oxide (NO), and (ROS) reactive oxygen species such as ozone (O_3), hydroxyl radicals (OH^-), atomic oxygen (O) generated during cold plasma treatment lead to breaking the bonds of peptidoglycan structure, initiate the lipid oxidation and oxidative modification of the membrane protein which destroys the bacterial cell wall membrane, eventually leads to death (Gavahian et al., 2019). The level of inactivation depends on the resistance of the culture to the treatment conditions.

4.3.1.2. Inactivation during plasma-activated water tempering (B)

PW tempering (B) achieved the reduction of 0.18, 0.27, 0.32, 0.19, 0.37, 0.39, 0.22, 0.31, 0.39 log cycles at 20 kV/20 min, 20 kV/40 min, 20 kV/60 min, 25 kV/20 min, 25 kV/40 min, 25 kV/60 min, 28 kV/20 min, 28 kV/40 min, 28 kV/60 min respectively. The PW tempering did not show a significant difference in achieving the log reduction of *Salmonella* under different cold plasma voltage conditions and time duration. It is due to the effectiveness of PW is increases by extending the plasma activation time over the water (Wang & Salvi, 2021). The antimicrobial activity of the PW tempering is due to the oxidative stress on the cell membrane of the bacterial cell through the oxidation-reduction potential (Rivera et al., 2024). Furthermore, a more acidic pH of the PW leads to decreases in the intracellular pH, below a certain threshold limit of the pH decline, leading to cell damage and resulting in death (Suo et al., 2022). Some studies focus on the effect of PW tempering on the grains. Suo et al. (2022) found that bran, low-grade flour, and patent flour from plasma-activated water tempered wheat grain have achieved the 0.51, 0.41, and 0.24 log reduction of total plate count through tempering the grain with PW (24 V /40 min). Rivera et al. (2024) found that PW tempering of wheat grain achieved a 1-log reduction of the *E. coli* in the wheat grain. The effectiveness of the inactivation with the PW tempering depends on the amount of dispersion of plasma-activated radicals into the water. The more radicals lead to greater activation (Ezzati et al., 2025). The concentration of the radicles varies with the type of equipment and operating conditions.

4.3.1.3. Inactivation during plasma treatment on flour (C)

Cold plasma treatment on the flour (C) achieved the log reduction of 0.19, 0.25, 0.32, 0.40, 0.56, 0.66, 1.12, 1.17 and 1.20 at 20 kV/20 min, 20 kV/40 min, 20 kV/60 min, 25 kV/20 min, 25 kV/40 min, 25 kV/60 min, 28 kV/20 min, 28 kV/40 min, and 28 kV/60 min respectively. The cold plasma

treatment on the flour was most effective at the highest voltage (28 kV) than at the lower voltage (20 kV). Through exposing the flour to the cold plasma, a significant log reduction was achieved initially at 25 kV/40 min. At 28kV, all treatment conditions achieved a similar log reduction without a significant difference. The microorganism exhibits a biphasic inactivation pattern through rapid initial kill followed by the slower phase (Ezzati et al., 2025). Lee et al. (2022) found that cold plasma treatment on the wheat flour with three gases, argon, air, and nitrogen, achieved the 2.66, 4.21, and 5.55-log reduction for the *E. coli*. Bahrami et al. (2016) treated the wheat flour with cold plasma; they did not see any change in the aerobic plate bacterial count and mold count. Soleimani et al. (2025) obtained a 4-log reduction of *Coliforms* with cold plasma treatment of wheat flour. Treatment of the grain is more effective at higher voltage than the flour, through achieving a log reduction of 1.403. Cold plasma is the surface treatment; it will not penetrate inside the grain. Still, it achieves the highest log reduction than the flour due to the barrier properties, such as the high area and agglomeration, which will reduce the efficiency of the cold plasma treatment and require a longer exposure duration (Staric et al., 2023; Zare et al., 2022). At the lower voltage, a similar log reduction is shown in the tempering, treatment with flour and grains. The amount of reduction obtained through the tempering and individual treatments is lower than in the previous studies. It is due to the decrease in the short-lived reactive species in the generated plasma (Rivera et al., 2024).

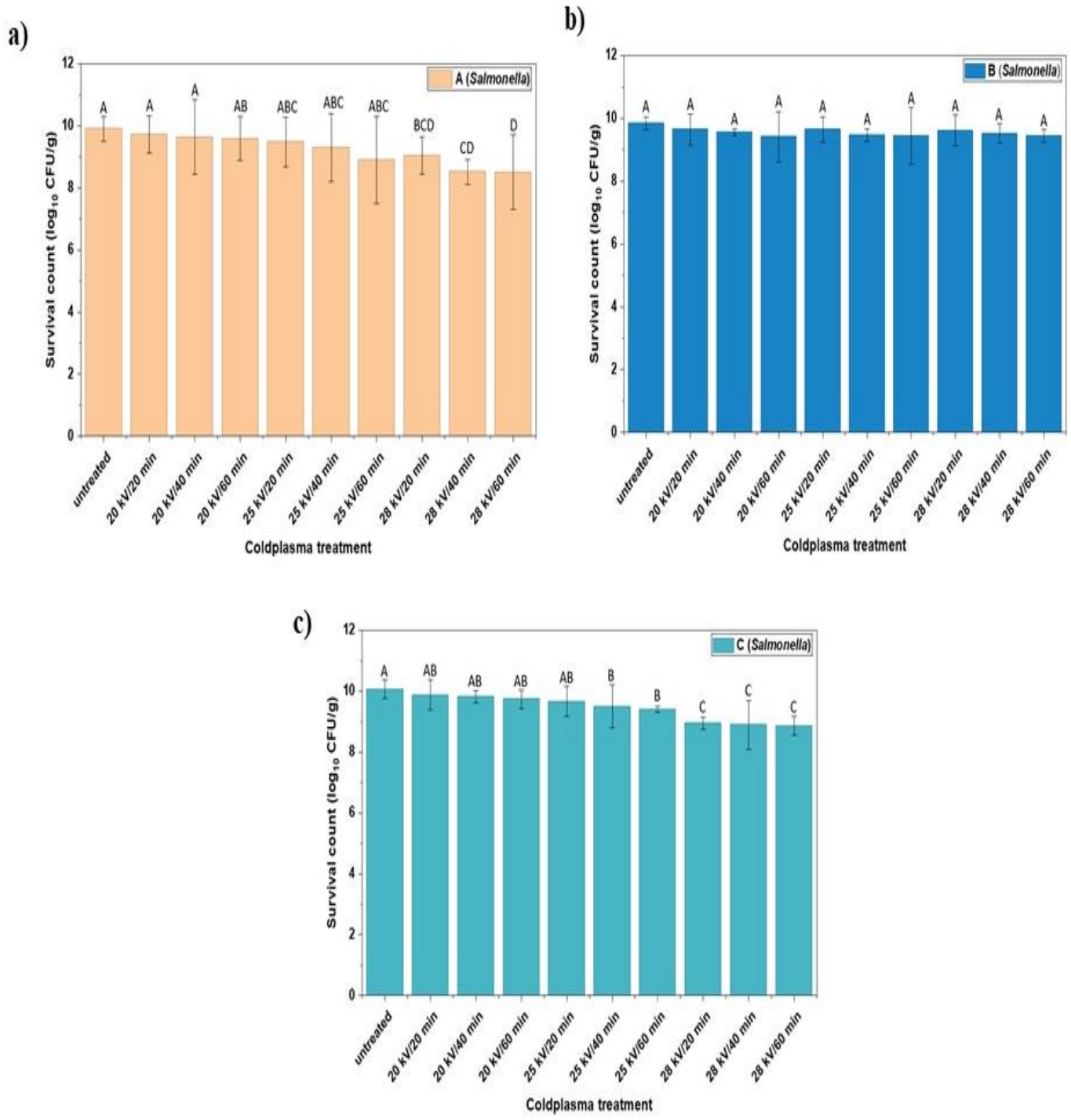

Figure 4.2: *Salmonella* inactivation during a) cold plasma treatment of grains (A), b) plasma-activated water tempering (B), c) cold plasma treatment of flour (C)

4.3.2. *Salmonella* inactivation during the combination of PW tempering, cold plasma treatment on grain and flour

Figure 4.3 (a), (b), (c) explains the effect of the sequence of the cold plasma treatment on the grain + PW tempering (A+B), cold plasma treatment on the flour + PW tempering (B+C), and cold plasma treatment on the grain + flour (A+C) on *Salmonella*.

4.3.2.1. Inactivation during treatment of A+B

Cold plasma treatment on the grain followed by PW tempering of the grains (A+B) leads to a reduction of 0.37, 0.54, 0.74, 0.61, 0.98, 1.41, 1.08, 1.70 and 1.80 log cycles at 20 kV/20 min, 20 kV/40 min, 20 kV/60 min, 25 kV/20 min, 25 kV/40 min, 25 kV/60 min, 28 kV/20 min, 28 kV/40 min, 28 kV/60 min respectively. The highest reduction obtained at 28 kV/60 min and the lowest log reduction at 20 kV/20 min have the 1.801 and 0.373 log CFU/g, respectively, which is similar to the individual treatment. Through the individual treatment of the A and B highest reduction obtained at the 28kV/60 min is 1.40 and 0.39, respectively. The lowest reduction achieved at 20kV/20 min for A and B are 0.18 and 0.18, respectively. The highest voltage and time duration lead to a greater reduction of the microorganisms. At the higher voltage inputs, the gas ionization and generates more significant quantities of the reactive species. These reactive species interact more effectively with the microbial cells, improving the inactivation efficiency (Ezzati et al., 2025). Amiri et al. (2025) found that higher voltage and exposure time of the cold plasma treatment of the powder lead to greater microbial reduction. Wang et al. (2022) treated the tilapia fillets with the cold plasma treatment and found that inactivation of the bacteria is time-dependent.

4.3.2.2. Inactivation during treatment of B+C

PW tempering followed by cold plasma treatment of the flour (B+C) which is milled by the lab scale grinder achieves the log reduction of 0.38, 0.52, 0.75, 0.59, 0.94, 1.06, 1.34, 1.48, 1.6 log

CFU/g at 20 kV/20 min, 20 kV/40 min, 20 kV/60 min, 25 kV/20 min, 25 kV/40 min, 25 kV/60 min, 28 kV/20 min, 28 kV/40 min, 28 kV/60 min respectively. 1.6 log CFU/g is the highest log reduction achieved at 28 kV/60 min, and 0.38 log CFU/g is the lowest log reduction achieved at 20 kV/20 min. The combination of the B+C have the lowest level of microbial reduction than the other combination such as A+B and A+C. It is due to the greater effectiveness of the individual treatment on the grains followed by the flour, and least through the PW tempering. Bahrami et al. (2016) found that treating the wheat flour with a low level of cold plasma did not change the total aerobic bacterial count or total mould count.

4.3.2.3. Inactivation during treatment of A+C

Cold plasma treatment of the grain followed by the flour (A+C) at 20 kV/20 min, 20 kV/40 min, 20 kV/60 min, 25 kV/20 min, 25 kV/40 min, 25 kV/60 min, 28 kV/20 min, 28 kV/40 min, 28 kV/60 min leads to log reduction of the 0.37, 0.52, 0.64, 0.82, 1.17, 1.67, 1.98, 2.56 and 2.60 log CFU/g respectively. A+C is more effective and achieved the highest log reduction (2.60 log CFU/g at 28 kV/60 min) than the A+B and B+C. It is due to the direct exposure of the cold plasma to the grain and flour are more effective that the PW tempering. PW is rich in ROS and RNS, which creates an acidic condition to enhance microbial inactivation synergistically with oxidative species (Le et al., 2023). Instead of the PW tempering, direct exposure to the cold plasma treatment produces the highly (ROS), and reactive nitrogen species (RNS) that directly interact with the grain surface, leading to oxidation, cell membrane damage eventually microbial inactivation (Charu et al., 2024).The highest log reduction through individual treatment of the grain is 1.40 log CFU/g at 28 kV/60 min. Meanwhile, through combined treatment, we can achieve similar and higher log reduction at short duration and lower voltage conditions, such as 1.41 at 25 kV/60 min, 1.48 at 28 kV/40 min, and 1.67 at 25 kV/60 min for the A+B, B+C, and A+C, respectively. The reduction

through combined treatment is much higher than the individual treatments. However, it is a time-consuming and energy-consuming process than the individual treatments.

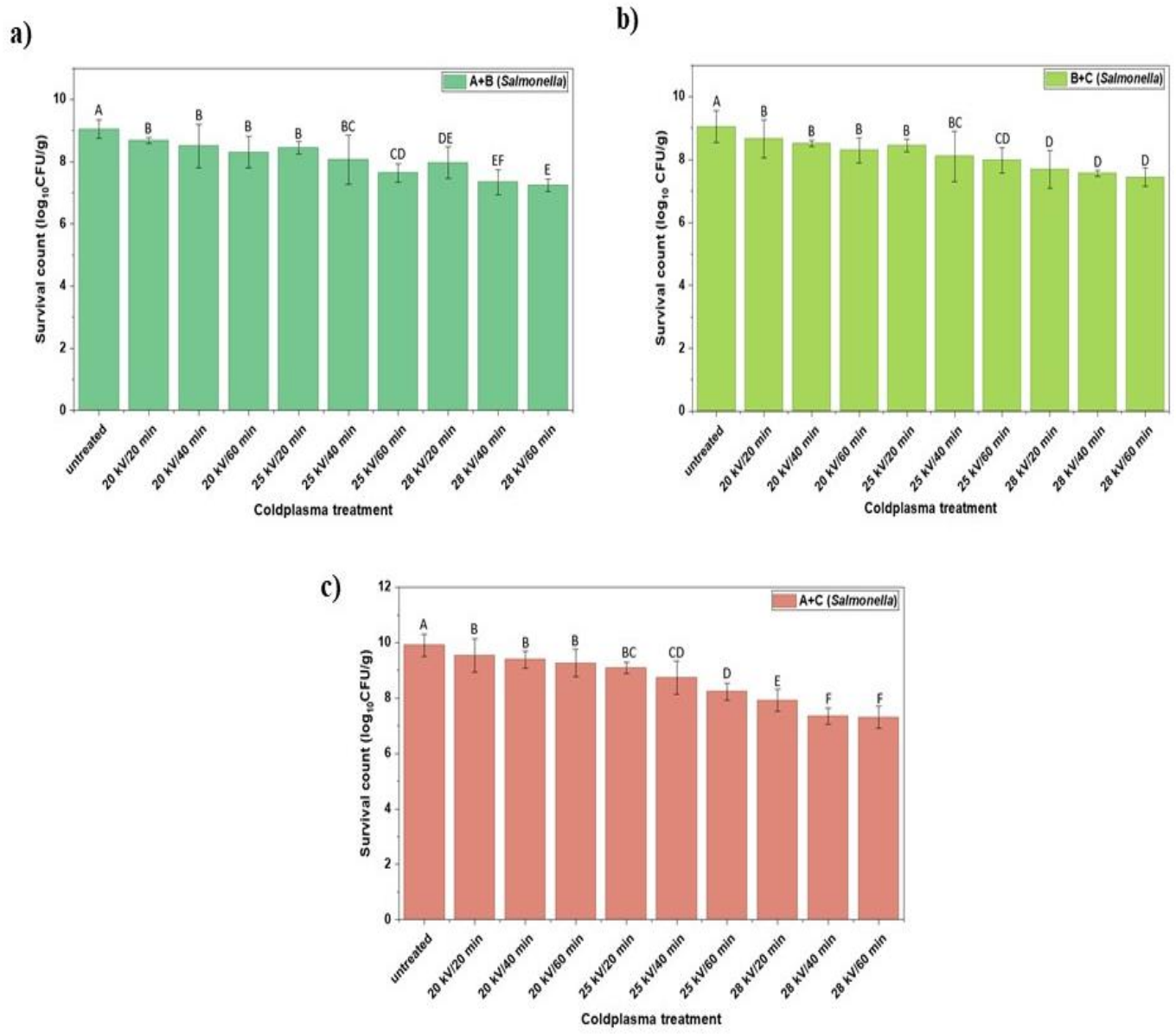

Figure 4.3*:* *Salmonella* inactivation during a) cold plasma treatment on the grain + PW tempering (A+B), b) cold plasma treatment on the flour + PW tempering (B+C), c) cold plasma treatment on the grain + flour (A+C)

4.3.3. *Salmonella* inactivation during the combined treatment of plasma-activated tempering, cold plasma treatment on grain and flour

Figure 4.4 (a)(b)(c) explains the log reduction of *Salmonella* with the combined treatment of PW tempering, cold plasma treatment of the grain and flour (A+B+C). The effect of A+B+C leads at 20 kV/20 min, 20 kV/40 min, 20 kV/60 min, 25 kV/20 min, 25 kV/40 min, 25 kV/60 min, 28 kV/20 min, 28 kV/40 min, 28 kV/60 min leads 0.56, 0.79, 1.06, 1.01, 1.54, 2.07, 2.20, 2.87, 3.00 log CFU/g log reduction of the *salmonella* respectively. The highest log reduction was achieved through a combination of treatments of A +B+C, which is 3.00 log CFU/g at 28 kV/60 min, and the lowest log reduction was 0.56 log CFU/g at 20 kV/20 min. No study was found on the inactivation of Salmonella in pearl millet. Meanwhile while some studies explored the effect of the thermal treatment on the microbial safety of the pearl millet. Charu et al. (2024) found that cold plasma treatment at 2 kV/5 min achieved the 5.82, 4.47, 3.47, and 3.6 log reduction in the pearl millet for the aerobic mesophiles, yeast, coliforms, and Enterococcus, respectively. Some of the studies conducted on the efficacy of the thermal treatment to achieve microbial inactivation in the pearl millet grains. Dikkala et al. (2018) got a 0.28 log cycle reduction of fungi through the heat treatment (150-170 ℃/ 1.5 min) and achieved the 1.25 CFU/g of the log reduction of fungi with combined treatment of heat and irradiation (2.5kGy). Naryanan and Ravi Kumar (2017) achieved a 4.69 CFU/g of the log reduction for the aerobic mesophile through roasting (180 ℃/10 min) in the Pearl millet. Choudhary et al. (2025) found that blanching (98 ℃/30 s followed by drying 50 ℃/1 hr) achieved the 4.71, 4.64, 4.47, and 3.09 log CFU/g of log reduction for the aerobic mesophiles, yeast, Coliform, and *Enterococcus*. Both the thermal and non-thermal technologies achieve a similar amount of log reduction in the pearl millet. The efficiency of the treatments varies with the resistance of the microbial nature.

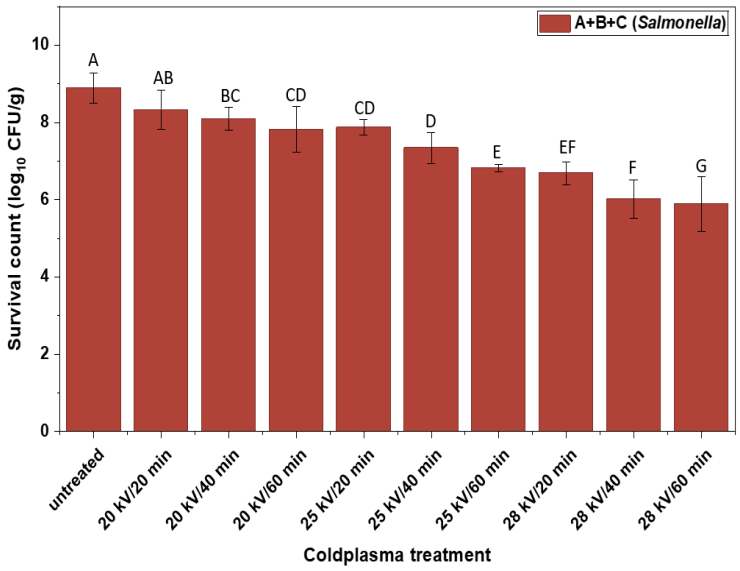

Figure 4.4*:* *Salmonella* inactivation during combined treatment of PW tempering, cold plasma treatment of the grain and flour (A+B+C)

4.3.4. Influence of cold plasma treatment on the physicochemical properties

The bulk, true, and tapped density of the flour in all conditions did not show any significant difference among all conditions except for the treatment A+B+C (522.2, 1468.7, 573.2 kg/m^3). It has the lowest bulk, true, and tapped density of flour among the remaining conditions (Table 4.1). It is due to the tempering that leads to the penetration of water into the kernels, which increases the weight of the flour particles at a slower rate than the volumetric expansion of the particles (Yoganandan et al., 2021). Bulk, true, and tapped density of the are 545.1, 1493.2, and 585.8 kg/m³, respectively, for untreated, Treatment A (542.3, 1495.8, and 582.3 kg/m³) for Treatment A, and A+C (543.5, 1494.3, and 583.4 kg/m³) for A+C. The color profile of the PMF is did not show any difference in the untreated (L^*=74.26, a^*=1.47, b^*=12.56), (L^*=74.01, a^*=1.50, b^*=12.58), and

A+C (L^*=74.1, a^*=1.44, b^*=12.59), but the color profile changed in the treatment A+B+C condition (L^*=72.91, a^*=1.21, b^*=11.35). Due to the tempering, the flour has a lighter shade than the untreated and cold plasma-treated flour. Tyl et al. (2019) found that tempered krenza flour has a lighter shade compared to untempered flour due to the increasing moisture content, toughening the outer layer of bran and softening the endosperm, leading to better separation and reducing the bran specks in the flour. Zhang et al. (2025) observed that tempered wheat grain has a higher whiteness value than normal wheat flour. The geometric mean diameter of the flour is higher for treatment A+B+C (284.3 μm) than for the untreated (250.2 μm), treatment A (248.2 μm), and treatment A+C (255.2 μm). An increase in the moisture content in the flour leads to an increase in the d_{gw} value (Curti et al., 2021). At high moisture content, water supports the formation of the liquid bridges. These bridged agglomerates behave like the large particles, which will eventually increase the d_{gw} value (Jung et al.,2018).Water activity and moisture content of the flour are decreased in treatment A (0.37,11.4%) and treatment A+C (0.35,11.1 %) due to the cold plasma treatment. Treatment A and treatment A+C both conditions water activity and moisture content are statistically significant. It is due to the exposure to the cold plasma, which leads to the breakdown of the water molecules to the simple oxygen free radicles. Charu et al. (2024) and Naraharasetti et al. (2025) found a similar trend of reducing the moisture content from 5.65% to 4.17% and from 7.3% to 7.1% after cold plasma treatment of the PMF. Treatment A+B+C has the highest moisture content (15.7%) and water activity (0.69) among the remaining conditions is due to the tempering process. Cold plasma and tempering did not show any significant difference ($p < 0.05$) in the protein and ash content of the PMF in all conditions.

Table 4.1: Effect of the cold plasma treatment and plasma-activated water tempering on the physical properties of Pearl millet flour

Attributes	Untreated	Treatment A	Treatment A+C	Treatment A+B+C
Moisture (%)	12.61 ± 0.3^{a}	11.4 ± 0.2^{b}	11.1 ± 0.1^{b}	15.71 ± 0.5^{c}
Protein (%)	8.59 ± 0.08^{a}	8.41 ± 0.09^{b}	8.56 ± 0.02^{ab}	8.49 ± 0.05^{ab}
Ash (%)	2.32 ± 0.3^{a}	2.30 ± 0.2^{a}	2.41 ± 0.2^{a}	2.35 ± 0.1^{a}
d_{gw} (μm)	250.2 ± 3.2^{a}	248.2 ± 8.2^{a}	255.2 ± 4.5^{a}	284.3 ± 2.4^{b}
Water activity	0.48 ± 0.03^{a}	0.37 ± 0.04^{b}	0.35 ± 0.01^{b}	0.69 ± 0.05^{c}
Bulk density (kg/m^3)	545.1 ± 2.5^{a}	542.3 ± 1.2^{a}	543.5 ± 1.9^{a}	522.2 ± 3.4^{b}
True density (kg/m^3)	1493.2 ± 2.2^{a}	1495.8 ± 1.8^{a}	1494.3 ± 1.5^{a}	1468.7 ± 3.2^{b}
Tapped density (kg/m^3)	585.8 ± 2.3^{a}	582.3 ± 1.8^{a}	583.4 ± 3.4^{a}	573.2 ± 1.5^{b}
Color profile				
*L**	74.26 ± 0.2^{a}	74.01 ± 0.19^{a}	74.1 ± 0.15^{a}	72.91 ± 0.1^{b}
*a**	1.47 ± 0.04^{a}	1.50 ± 0.01^{a}	1.44 ± 0.05^{a}	1.21 ± 0.01^{b}
*b**	12.56 ± 0.04^{a}	12.58 ± 0.03^{a}	12.59 ± 0.1^{a}	11.35 ± 0.1^{b}

The dissimilar alphabets (a & b) in the superscripts denote that the mean values are statistically different across the treatment conditions at $p < 0.05$.
Abbreviations: A, cold plasma treatment on the grains; A+C, cold plasma treatment on the grain + flour; A+B+C , combined treatment of PW tempering, cold plasma treatment of the grain and flour.

4.4. Conclusion

Cold plasma treatment of the grains, followed by PW tempering and cold plasma treatment of the grains (A+B+C), helps to achieve a 3-log reduction of *Salmonella*. Individual treatment achieves the 1.4 log reduction, and combined treatment of (A+C) helps achieve the 2.6 log reduction. Results obtained after 28 kV/60 min of treatment show a positive log reduction. However, some limitations need to be addressed. The overall combined treatment is not feasible to do on an industrial scale to achieve the 3-log reduction. It is an energy and time-consuming process. Further research should focus on the resistance of the *salmonella* to longer exposure duration at high

voltage of the cold plasma treatment and the efficacy of the cold plasma technology on other pathogens in the pearl millet processing.

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Chapter -5

Influence of milling methods and cold plasma treatment on the functional and baking properties of the pearl millet flour

Abstract

Pearl millet is a crop with excellent resilience to survive under harsh environmental conditions. pearl millet is gluten-free, and it is hard to mimic the wheat bread textural attributes. Cold plasma is a non-thermal technology that helps to enhance the viscosity profile and functional properties of the cereal flour. The objective of the study is to select its milling methods through pin mill and hammer mill and determination of dough rheological properties. Furthermore, the formulation of a functional bread was optimized using mixture design while utilizing the cold plasma-treated and tempered pearl millet flour (PMF). Pin-milled PMF has a uniform particle distribution, and tempered pearl millet flour exhibited the highest yield, 93.5%. Varied proportions of the wheat flour (70-87 g), pearl millet flour (10-27 g), and shortening agent (3-6 g) were used to assess the bread hardness, crust color, and specific volume, as per D-optimal mixture design. A special cubic model was fitted to each response in both conditions. An optimized blend for the tempered flour composed of 85.5 g refined wheat flour,11.5 g PMF, and 3 g of shortening, having the hardness, total color change of crust, and specific volume 6.06 N, 5.47, and 2.82 $mL.g^{-1,}$ respectively. Similarly, the untempered pearl millet flour has 75.6 g wheat flour, 18.3 g PMF, and 6 g of the shortening agent, comprising the hardness, total color change of crust, and specific volume 7.32 N, 6.60, and 3.04 $mL.g^{-1}$ respectively. Cold plasma treatment of PMF at 25 kV/8 min enhances the overall functional properties and texture attributes of the bread.

5.1. Introduction

Pearl millet (*Pennisetum glaucum (L.) R.Br.*) is one of the major millets belonging to the Poaceae family and the small-grain C4 tropical cereal grass cultivated widely in arid and semi-arid regions. Dry conditions, low pH, High temperature, and salt content are favourable to the growth of the pearl millet (Taylor, 2016). Pearl millet has the 6.5-12.4% moisture,1.6-3.1% ash,2.7-7.1% fat,8.5-15.1% protein,1.2-4.0% crude fiber, and 58.5-72.5% carbohydrate. It is a high-energy cereal having a good amount of unsaturated fats, essential amino acids, a lower glycemic index (54-68), and higher resistant starch (8-12%) and dietary fiber (11-13%), which is the ideal food for people suffering from diabetes and cardiac disease. Additionally, it acts as an anticarcinogenic agent (Naraharasetti et al., 2025a).

The United Nations declared that climate change is a global emergency. The world needs sustainable resources to feed the future population (United Nations Global Compact, 2025). There is a growing demand for the pearl millet grain due to its enriched nutritional and resilient properties, which will be an alternative source for future generations. Moreover, pearl millet is consumed as the whole grain flour because it has a pack of dense nutrient sources in the outer layer of the bran and germ (Meena et al., 2024). Tempering is the process of adding a small amount of moisture content to the grain, which is the processing step to wheat in the milling operation. Tempering helps to achieve uniform distributions of nutrients in the grains, improving milling yield, reducing the breakage through softening endosperm and toughening bran (Tyl et al., 2019). Apart from that, tempering helps for the uniform particle size distribution, reduces the damage starch content and the energy required for the milling process (Kweon et al., 2009). Followed by milling method and conditions have a great influence on the flow and baking properties of the flour. Some studies explored the effect of milling on the pearl millet properties. Benhura. (2023)

& Tiwari et al. (2022) studied the effect of commercial and traditional milling on the nutritional and physical properties of pearl millet products. Sidhu et al. (2016) explore the influence of milling speed on the proximate composition of the pearl millet flour. So far, no study has explored the significance of tempering and the effect of different milling conditions on the physical properties of pearl millet flour. As well as it is challenging to develop bread with PMF, which ideally has the textural characteristics made with whole wheat flour due to its gluten-free nature.

Some studies explored the pearl millet-bread making and optimization with the composite wheat flour. Maktouf et al. (2016) evaluated the dough rheological properties and bread texture of the pearl millet-wheat flour mix. de Aguiar et al. (2024) explored the development of whole-grain pearl millet-based gluten-free bread while considering both nutrition and sensory features. Ranasalva et al. (2014) developed bread from the fermented pearl millet flour. Nehra et al. (2021) studied the effect of incorporating different proportions of pearl millet flour on the bread quality and sensory characteristics. No study has explored the effect of the non-thermal technology on the viscoelastic and baking properties of the PMF.

Cold plasma technology, having a non-thermal state of matter generated by gases subject to electrical discharges, produces reactive species such as reactive oxygen and reactive nitrogen species (Prakash et al., 2023). It helps to enhance the baking properties through partial depolymerization and cross-linking of the starch granules, which helps enhance the strength and elasticity of the dough (Jaddu et al., 2024). Limited research has examined the effect of cold plasma on the functional and baking properties of flour. In line with this, Sarkar et al. (2023) found that cold plasma treatment 30 kV/20 min helps for the product formation through exhibiting high water absorption capacity, oil absorption capacity, emulsifying capacity, and foaming capacity. Additionally, it has a higher storage modulus value, indicating an elastic response. Gebremical et

al. (2025) studied that cold plasma treatment (15 kV/5-30min) of gluten-free teff flour leads to improving its techno-functional properties by increasing the swelling, solubility, water absorption capacity, and starch damage, which helps to produce the sticky dough during mixing with water-soluble compounds. Chaple et al. (2020) observed that cold plasma treatment (5-30 min/80 kV) of whole wheat flour helps to improve the functionality by increasing flour hydration properties, final and pasting viscosities. Menkovska et al. (2014) found that bread made with cold plasma-treated wheat flour (1000 ppm at 2.5 L/min) has a brighter, creamy color, improved gluten quality, dough stability, and strength. The loaves made with treated flour have expansion in the total and specific volume, along with an enhancement in appearance and porosity structure.

Regardless of the number of studies on the pearl millet grains, no study explained the effect of the different milling methods and cold plasma treatment on the tempered and untempered grains mixing and baking properties . Further developments are needed to help enhance the utilization of PMF in industrial applications. The objective of this study is to explain the significance of different milling methods and tempering on the pearl millet flour, dough, and baking properties with and without the application of the cold plasma treatment. Initially, an optimized milling method and cold plasma treatment condition were selected to determine the rheological properties of the dough and final baked product characteristics. This study offers a comparison of the baking properties of bread made with 100% refined wheat flour and the optimized conditions from the optimal mixture design.

5.2. Materials and Methods

5.2.1. Pearl millet grain

The pearl millet (*Pennisetum glaucum*), used for this study, was procured from Ancient Wisdom Organic Bajra Grain, Mumbai, Maharashtra, India which is having moisture content of 11.6±0.7%.

5.2.2. Experimental design

A 2^2-full factorial design (two factors and two levels) was used for the initial stages of the milling study. Pearl millet grain (tempered and untempered) was milled through two different milling techniques, viz., pin mill (PM) and hammer mill (HM). These two milling techniques were selected from the preliminary study of different milling methods and quality analysis. A set of the physical properties, particle size distribution, and yield was analysed. These include true, tapped, bulk density, and volume surface mean diameter. Furthermore, the optimized milling condition, which has the greatest yield and uniform particle size distribution, was used for the next step in analyzing the mixing and pasting properties of the PMF with and without cold plasma treatment at 25 kV/8 min which inactivates the more than 90% of the enzymes such as the lipase, lipoxygenase, peroxidase and polyphenol oxidase (Naraharasetti et al., 2025b). The treatment condition that exhibited the desirable mixing and pasting properties was further taken for the optimization of the bread formulation of PMF. For making the optimization of the bread D-optimal mixture design was used, which has a total mixture of ($X_1 + X_2 + X_3$) 100 g. The level of the refined wheat flour (X_1: g), PMF (X_2: g), and shortening (X_3: g) was varied at each condition. The responses analyzed were hardness (Y_1, N), total color change (ΔE^*) of crust (Y_2, -), and specific volume (Y_3, $mL.g^{-1}$). A total of 17 trials were conducted across the study. Additional baking properties, such as C-cell analysis and TPA (texture profile analysis) were conducted for the optimized conditions.

5.2.3. Tempering

Pearl millet grains were tempered with a predetermined amount of distilled water to reach a moisture content of 16 to 18% (w.b) while conditioning them for 24h as detailed in AACC Method 44-40.01 (Rivera et al., 2022). The moisture content was chosen based on the wheat tempering

method, which is used for the uniform moisture distribution, maximum equipment efficiency, and optimum particle size and rheology (Yoganandan et al., 2021). Tempered samples were hand mixed and shaken for the uniform distribution of moisture content in the pack. samples were tightly air-packed in zip-lock bags and held at room temperature for 24 hours.

5.2.4. Milling

1.5 kg of the tempered and untempered pearl millet grains each were individually milled using the hammer mill**,** which runs at 3,000 rpm (GlenMills Inc., Clifton, NJ, USA type: SK300;1100 W, 5 A), and pin mill (model UT-03, Bauermeister, Inc., Memphis, TN). The milling parameters of each mill were optimized by a preliminary study for the maximum recovery of the whole pearl millet flour. The target for the particle size of the flour was set below 212 μm, which aligns with code of Federal Regulations for the wheat flour (FDA, 2021). The milling yield was calculated using the Eq. 1.

$$\text{Yield (\%)} = \frac{\text{Weight of the flour after milling (g}}{\text{Initial weight of the grain (g)}} \times 100 \qquad (1)$$

5.2.5. Density

True, bulk, and tapped density were measured for the tempered and untampered pearl millet flour (PMF) from each milling method. Bulk density was measured using the Winchester cup arrangement (Seedburo Equipment, DesPlaines, IL, USA). True density of the sample measured using the gas pycnometer (AccuPync II 1340, Micromeritics, Norcross, GA, USA). Tapped density was measured using the Autotap Density Analyzer (Quantachrome Instruments, Boynton Beach, FL, USA). All the density calculation was done using the formula mentioned by Pulivarthi et al. (2022).

5.2.6. Particle size distribution

The particle size distribution of the pearl millet flour was analyzed by using a Ro-Tap Sieve shaker (Hosokawa Micron, E20). 100g of tempered and untempered pearl millet flour from each milling method was sieved using the series of sieves in the descending order of 595, 420, 297, 210, 149, 105, 74, 53, and 44 μm, respectively. The volume-surface mean diameter is also known as the Sauter mean diameter ($D_{3,2}$), of the pearl millet flour was calculated using Eq . 2.

$$D_{(3,2)} = \frac{\sum m_i \cdot d_i^3}{\sum m_i \cdot d_i^2} \qquad (2)$$

m_i = mass fraction of particles in each sieve

d_i = diameter of each sieve (μm)

5.2.7. Dough Properties

5.2.7.1. Mixing properties

The mixing behaviour and water absorption capacity of the flour from different milling were studied using the Chopin S protocol at a base of 14% using the Mixolab instrument (Chopin, France). The physico-chemical behaviour of the dough made with PMF was studied by measuring the torques (Nm) produced during mixing by gradually increasing the temperature to 30 °C for 30 min with the mixing speed of 80 rpm of target torques of 1.100 Nm with maintain the constant speed through the experiment.

5.2.7.2. Pasting properties

A rapid visco analyzer (RVA 4500, Perten Instruments, Waltham, MA, USA) was used to determine the pasting properties of samples. The samples were prepared by combining the pearl millet flour with distilled water to create a 15% d.b. (dry basis) suspension. The RVA test parameters were set according to the heating and cooling procedure of AACC Method 76–21 (Rajpurohit et al.,2025).

5.2.8. Baking and quality analysis of bread

Pearl millet flour was baked using the AACC method 10-10.03, with the baking ingredients consisting of a baker's percentage of 100% flour, 3.0% yeast, 6.0% sucrose, 1.5% salt, water (based on mixolab absorption), and 3.0% shortening. flour, dry and wet ingredients are mixed in a mixer for 9 min. After rounding and fermenting dough went through the sheeting and molding, followed by resting for 5 min. The molded dough was placed in the grease bake pan, proofed (30-35 ℃, 95% RH), and baked in the oven (215 ℃, 10 min). Post-baking, bread is cooled to reach room temperature. The specific volume (cm^3/g) were measured using the laser topography (Volscan Stable Micro Systems, Godalming, Surrey, United Kingdom). The bread loaves were sliced, having a thickness of 15 mm. C- cell analysis was performed using the C-cell image analyser (CCFRA Tech Ltd., Warrington, UK) to measure slice area (mm^{-2}), number of cells, area of cells (%), cell to area (cm^{-2}), wall thickness (mm), and cell diameter (mm). The crust and crumb colors were measured using the colorimeter (MiniScan EZ 4000 Portable Spectrophotometer (Hunter Lab, Reston, VA, USA). Furthermore, texture profile analysis was conducted using the TA-XTPlus texture analyser (Stable Microsystems, Godalming, UK) to measure the hardness (N), resilience (%), cohesion, springiness (%), gumminess (N), and chewiness (N). All the quality analysis of bread were measured in triplicate.

5.2.9. Statistical analysis

All the milling and baking experiments are conducted and analysed in triplicate. The milling and baking data were analyzed through analysis of variance (ANOVA) using the Origin Pro 2023b software (Origin Lab Corporation, Northampton, MA, USA) and Microsoft Excel. Design Expert 7.0 software (StatEase, USA) was used for the optimal mixture design and response surface optimization.

5.3. Results and Discussion

5.3.1. Influence of tempering and milling on the grain properties

Some of the physical properties of tempered and untempered PMF using different milling methods have been summarized in Table 5.1. The hammer milled, untempered grain exhibited the highest bulk density (585.25 kg/m³), meanwhile, pin mill tempered grain exhibited the lowest bulk density (521 kg/m³). Milling method and tempering have a significant influence on the bulk density of the PMF. Tempering reduced the bulk density of the PMF in both pin-milled (tempered flour (521.77 kg/m³), untempered flour (542.19 kg/m³)) and hammer-milled flour (tempered flour (572.77 kg/m³), untempered flour (585.25 kg/m³)). Tapped density of flour also reduced with the tempering with incorporating the moisture into the grain. The tapped densities of the tempered and untempered flours are 594.76 kg/m^3(HM), 580.9 kg/m^3 (PM), and 604.87 kg/m^3 (HM), 585.819 kg/m^3 (PM), respectively. Similarly, true density also decreased with the tempering, such as the tempered flour 1482.12 kg/m^3 (HM), 1473.32 kg/m^3 (PM), and untempered flour 1508.22 kg/m^3 (HM), 1495.45 kg/m^3 (PM). A decrease in the bulk, tapped and true density of the tempered grains due to the penetration of the water into the kernel leads to an increase in the weight of the kernel at a slower rate compared to the volumetric expansion of the kernels. Yoganandan et al. (2021) reported similar findings, lowering the density of the flour with the tempering. Density (bulk, true, and tapped densities) obtained from the hammer mill (585.25 kg/m^3, 1508.22 kg/m^3, 604.87kg/m^3 (HMU), 572.77 kg/m^3, 1482.12 kg/m^3, 594.76 kg/m^3 (HMT)) is comparatively higher than the pin mill (542.19 kg/m^3, 1495.45 kg/m^3, 585.82 kg/m^3 (PMU), 521.77 kg/m^3, 1473.32 kg/m^3, 580.9 kg/m^3(PMT)). Higher density of hammer-milled flour is due to the production of a higher coarse particles than the more uniform particles in the pin mill (Scanlon et al., 2018). The milling yield for all conditions ranged from 87.3% to 93.5%. Hammer mill

untempered grain has the lowest yield of 87.3% and pin mill tempered grain has the highest yield of 93.5%. the hammer mill tempered and pin mill untempered grain have 89.1% and 91.5% respectively. Tempering of the grain in both milling conditions gave the highest yield than the untempered grain. tempering leads to the uniform distribution of the water in the grain the toughening the bran layer while plasticizing the endosperm, allowing the cleaner separation of the bran and endosperm and a higher flour extraction rate (Tyl et al., 2019; Warechowska et al., 2016). Hammer mill breaks the grain by the mechanisms of impact through striking the particles against the screen. pin mill applies the impact and shear forces together at high speed without screens. In the hammer mill, clogging or inefficient screens, coarse particles remaining on the screen will reduce the measurable yield of flour (Ammala, 2023; Zhang et al., 2025a). Kweon et al. (2009) found that wheat grain tempering (12% moisture content) increased the yield of the flour.

Table 5.1: Some of the physical properties of the tempered and untempered PMF using different milling methods. pinmill; hammer mill

Attributes	HMU	HMT	PMU	PMT
Volume surface mean diameter (μm)	319.99 ± 5.404^{a}	307.18 ± 8.77^{a}	221.92 ± 6.77^{b}	304.73 ± 0.202^{a}
Bulk density(kg/m³)	585.25 ± 2.1^{a}	572.77 ± 3.2^{b}	542.19 ± 4.8^{c}	521.77 ± 1.6^{d}
True density (kg/m³)	1508.22 ± 0.02^{a}	1482.12 ± 0.05^{b}	1495.45 ± 0.09^{c}	1473.32 ± 0.07^{d}
Tapped density(kg/m³)	604.87 ± 4.23^{a}	594.76 ± 3.52^{b}	585.819 ± 2.79^{c}	580.9 ± 1.22^{c}
Yield (%)	87.35 ± 0.9^{c}	89.16 ± 1.1^{bc}	91.5 ± 1.3^{ab}	93.5 ± 0.8^{a}

The dissimilar alphabets (a, b, c & d) in the superscripts denote that the mean values are statistically different across the treatment conditions at $p < 0.05$.
Abbreviations: HMU, hammer-milled untempered grain; HMT, hammer-milled tempered grain; PMU, pin-milled untempered grain; PMT, pin-milled tempered grain.

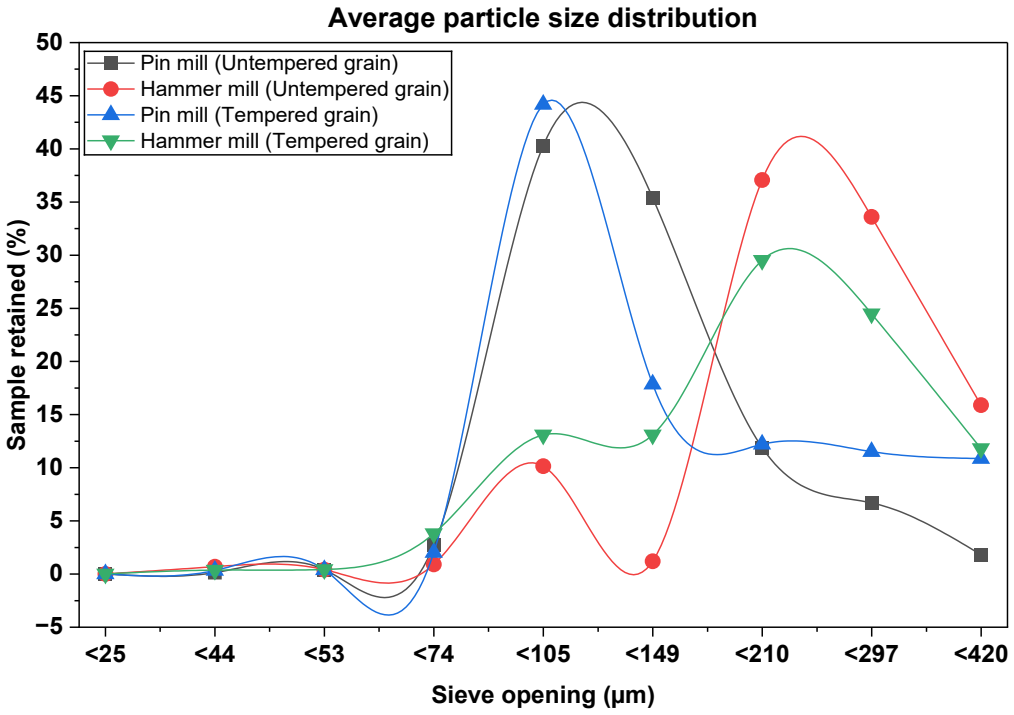
5.3.2. Influence of tempering and milling on particle size distribution of the flour

The volume surface mean diameter of the flour varied for the different milling techniques in the tempered and untempered grain (Table 5.1). Hammer milled untempered grain produced the coarsest flour (319.99 μm), followed by the hammer milled tempered grain (307.18 μm), pin milled tempered grain (304.73 μm), and pin milled untempered grain, which had the finest flour (221.92 μm). The particle size distribution of various flours from different milling techniques is explained in Figure 5.1.

For the Hammer mill tempered and untempered the maximum flour is retained on the 210 μm sieve with the mass fractions of 28.1%-33.4% and 38.8% – 43.08 % respectively. For the pin mill, the tempered and untempered maximum flour retained on the 105 μm sieve with mass fractions of 41.0%-47.3 % and 39.1%-46.7% respectively. The next best passes for the pin-milled flour are 149 μm with mass fractions of 37.7%-39.1% and 17.5%-20.9% of untempered and tempered flour, respectively. For the hammer-milled flour next best passes through the 105 μm with mass fractions of 24.8-31.5% and 24.8-30.3% for the untempered and tempered flour, respectively (Figure 5.1). The pin mill produced finer and more uniform particles than the hammer mill, which produces coarse particles. Pulivarthi et al. (2022) found a similar trend in the particle size analysis of Teff grain milled with different milling methods, such as the hammer, roller, and pin mill. Hammer-milled and pin-milled tempered and untempered flour exhibited the bimodal and unimodal particle size distribution, respectively (Figure 5.1).The unimodal distribution of pin-milled flour at <105 μm. The first and second modes of hammer-milled flour at <105 μm and <250 μm, respectively. There is a significant difference in the particle distribution between the tempering process in the hammer milling than the pin mill. Tempered flour has larger particles in

size than the untempered flour through the hammer milling. It is due the tempering plasticizes the endosperm and toughen the bran, making the milling process less aggressive and produce the lesser fine particles (Tyl et al., 2019). At the same time there is an inconsistency in the particle size distribution of the hammer-milled flour compared to the pin mill due to the high-speed impact of the hammer, producing flour with a broader and less uniform particle size distribution (Zhang et al., 2025a).

a)



b)

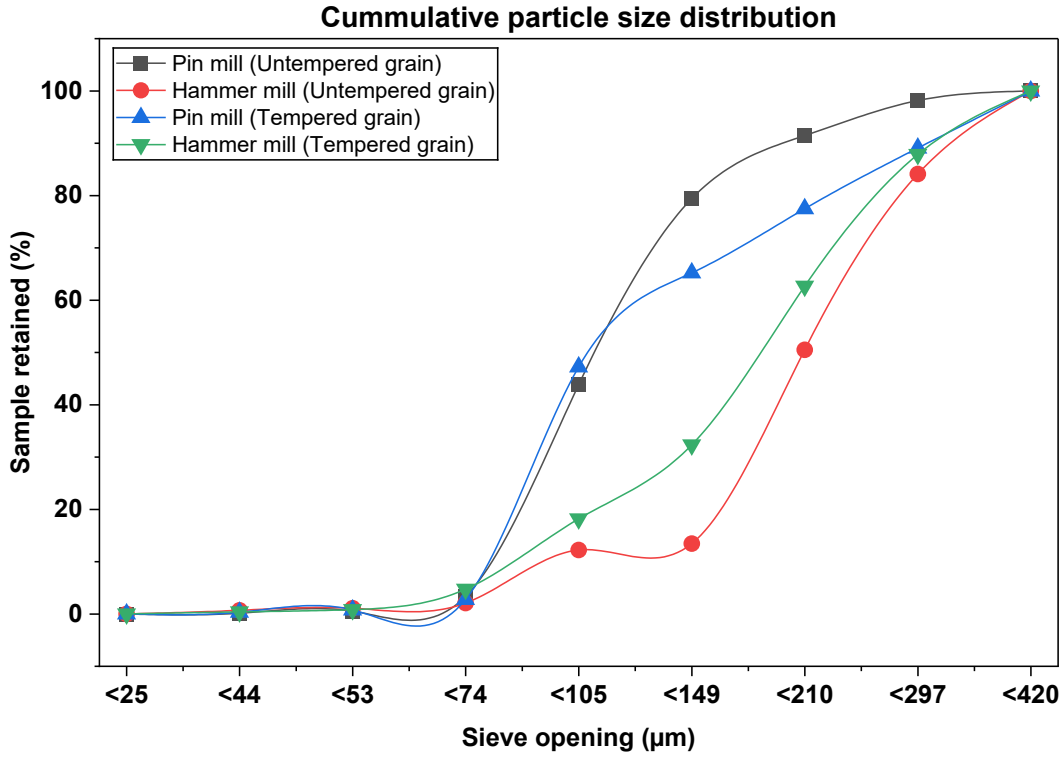


Figure 5.1: Average and cumulative particle size analysis of tempered and untempered PMF from different milling methods. pinmill; hammer mill

5.3.3. Pasting properties

RVA profile of the tempered, untempered, and cold plasma-treated PMF is presented in Table 5.2. PMT has a higher peak viscosity (1282 cP) than the PMU (1132 cP), indicating higher starch swelling capacity and PMT (1881 cP) has a higher final viscosity than the PMU (1819.3 cP). Final viscosity refers to the ability of the flour to form a viscoelastic slurry upon cooling (50 °C). The increase in final and peak viscosity due to tempering leads to the uniform distribution of water inside the grain, resulting in the swelling of the starch granules within the grain. This leads to a lower amount of damaged starch and a larger particle size in the milling process. Damaged starch will combine with water through intermolecular hydrogen bonds and small particles have more

surface area in contact with water molecules, leading to the reduction of the gelatinization (He et al., 2022). Breakdown viscosity indicates how much gelatinized starch granules collapse during continuous heating and stirring, leading to a reduction in viscosity. PMU (362.6 cP) has a higher breakdown viscosity than the PMT (319 cP). Adequate water in the tempering process leads to the rearrangement of starch chains, which strengthens the amorphous region of the starch and makes it hard to break down the starch granules in the tempered PMF (Lv et al., 2022). PMT has a higher setback viscosity (1025 cP) than the PMU (1014.3 cP), presenting higher retrogradation due to the tempering of the grain. Trough viscosity is the minimum viscosity measured during the holding stage at high temperature, which means the extent of starch granule disintegration. PMU (805 cP) has a lower trough viscosity than the PMT (856.6 cP). Tempered grain that is already well hydrated can swell more uniformly, forming a stronger, continuous network and holding the higher viscosity under various temperature conditions. Pasting temperature is the minimum temperature required to cook the flour, and peak time refers to the time taken for the flour to reach the maximum viscosity during the test under controlled heating. PMU and PMT did not show a significant difference ($p < 0.05$) in the pasting temperature and peak time of the flours. The cold plasma-treated untempered four (PMU+ CP_1) has a better viscosity profile than the PMU. Cold plasma-treated flour (PMU+ CP_1) has a higher peak, trough, breakdown, final, and setback viscosity that are 1360 cP, 982 cP, 431 cP, 2371 cP, and 1389 cP, respectively. There is no significant difference in the peak time and pasting temperature in between any samples such as the cold plasma treated, tempered and untempered conditions. The enhancement of the pasting profile of cold plasma-treated samples is due to the cross-linking of the starch by oxidation. The plasma reactive species lead to the loosening of the starch granules, permitting greater swelling and free radicals disrupt the hydrogen bonding, promoting the absorption of water molecules, resulting in the greater

viscosity (Chaple et al., 2020). Lokeswari et al. (2021) found that cold plasma treatment (40 kV/5-15 min) enhanced the trough and peak viscosity of the pearl millet flour. Pearl millet tempered flour (PMT) has the greatest viscosity profile compared to the cold plasma-treated tempered pearl millet flour (PMT + CP_1). The peak, trough, breakdown, final, and setback viscosity values are 1042 cP, 784 cP, 258 cP, 1767 cP, and 987 cP, respectively. Soaking the pre-hydrated starch granules makes them more susceptible to the plasma-induced reaction. During the cold plasma exposure, the generative plasma species lead to the starch molecular degradation, granule structure disruption of starch and protein, reduction of linear starch and relative crystallinity, leading to decreases in starch viscosity (Thirumdas et al., 2016 & Ma et al., 2024). At the same time, higher protein-water interactions may restrict the water available for the starch, thus leads to limiting viscosity and structural weakness (Gebremical et al., 2025). Based on the protein and starch fractions, availability of the moisture cold plasma treatment is showing a different trend. Gebremical et al. (2025) found that plasma treatment reduced the viscosity properties of the teff flour. Similar, Ma et al. (2024) and Chauhan et al. (2023) observed that cold plasma treatment reduced the viscosity of the wheat starch and proso millet starch, respectively.

Table 5.2: Pasting properties of the tempered, untempered, and cold plasma-treated PMF

Sample	Peak viscosity (cP)	Trough viscosity (cP)	Breakdown viscosity (cP)	Final viscosity (cP)	Setback viscosity (cP)	Peak time (min)	Pasting temperature
PMU	1132 ± 38.21^{b}	805 ± 23.51^{b}	362.6 ± 15.30^{ab}	1819.3 ± 24.3^{bc}	1014.3 ± 12.7^{b}	5.49 ± 0.03^{a}	80.6 ± 1.48^{b}
PMT	1282 ± 46.66^{a}	856.6 ± 79.47^{ab}	319 ± 51.403^{bc}	1881 ± 32.4^{b}	1025 ± 14.8^{b}	5.46 ± 0.06^{a}	82.88 ± 1.71^{ab}
PMT + CP_1	1042 ± 13.26^{c}	784 ± 19.26^{b}	258 ± 26.2^{c}	1767 ± 14.8^{c}	987 ± 21.5^{b}	5.56 ± 0.04^{a}	85.175 ± 1.35^{a}
PMU + CP_1	1360 ± 60.37^{a}	982 ± 47.73^{a}	431 ± 16.69^{a}	2371 ± 93.26^{a}	1389 ± 45.88^{a}	5.53 ± 0.07^{a}	80.81 ± 1.27^{b}

The dissimilar alphabets (a, b, c & d) in the superscripts denote that the mean values are statistically different across the treatment conditions at $p < 0.05$.
Abbreviations: PMU, pin-milled untempered grain; PMT, pin-milled tempered grain; PMT + CP_1, pin-milled

tempered grain +25kV/8min cold plasma treatment; PMU + CP_1, pin-milled untempered grain + 25kV/8min cold plasma treatment.

5.3.4. Mixing properties

The mixing properties of the tempered, untempered, and cold plasma-treated PMF are presented in Table 5.3. Pearl millet tempered (PMU) and untempered flour (PMT) have water absorption capacities of 52.8% and 50.9%, respectively. Cold plasma-treated pearl millet tempered (PMT + CP_1) and untempered (PMU + CP_1) flour have 51.7% and 53.5% of water absorption capacity, respectively. Soaking the grains in water during the tempering process reduces the water absorption capacity of the flour because the residual moisture in the grain will reduce the capacity to absorb the moisture. Chen et al. (2020) found a similar trend of reduction in water absorption capacity of the tempered wheat grain. In the meantime, the cold plasma-treated PMF has a higher water absorption capacity than the untreated PMF. It is due to the cold plasma treatment, which leads to a reduction in the moisture content of the flour by breaking down the water molecules into oxygen radicals (Naraharasetti et al., 2025b; Sarkar et al., 2023). The development time for the PMU, PMT, PMT + CP_1, and PMU + CP_1 is 2.1, 5.1, 2.0, and 8.1 min, respectively. Development time represents the time necessary to hydrate the whole sample. Suprabha Raj et al. (2023) found that development time and stability increased by tempering the wheat grains. However, we did not see an increase in the stability with the tempering of grains. PMT and PMT + CP_1 also did not show any difference ($p < 0.05$) in stability, such as the 1.5 min, 1.5 min, respectively. Softening was varied across all samples. The PMU and PMT have 0.18 nm and 0.167 nm, respectively. For the PMT + CP_1, PMU + CP_1 softening values are 0.231 nm and 0.123 nm, respectively. Mixing tolerance index for the PMU, PMT, PMT + CP_1, PMU + CP_1 are 0.23nm, 0.323, 0.453, 0.213, and Cmax values are 1.03 nm ,0.657 nm, 0.475 nm, 1.1 nm, respectively. Cold plasma-treated untempered flour has exhibited a greater mixolab profile compared to the

remaining samples, like the pasting profile. Suo et al. (2022) also found the similar trend of the reduction of the viscosity and mixing properties with the plasma activated tempering of the wheat grains. Cold plasma treated untempered flour have the higher mixing tolerance index than the remaining samples.This is due to the cold plasma generative reactive species that oxidize sulfhydryl groups in the proteins, promoting disulfide bond formation. This strengthens the protein network, requiring the longer mixing time and making the dough stronger (Bahrami et al., 2016). Misra et al. (2015) found that improvement in strength through cold plasma treatment by formation of disulphide bonds. Further study is needed to analyze the reason behind the reduction of the viscosity and mixing profile for the tempered cold plasma-treated pearl millet flour while compared to the untreated tempered pearl millet flour.

Table 5.3: Mixing properties of the tempered, untempered, and cold plasma-treated PMF

Sample	Water absorption (%)	Development time (min)	Stability (min)	Softening (nm)	Mixing tolerance index (nm)	Cmax (nm)
PMU	52.8 ± 0.3	2.1 ± 0.1	1.7 ± 0.4	0.18 ± 0.04	0.23 ± 0.00	1.03 ± 0.0
PMT	50.9 ± 0.05	5.1 ± 0.15	1.5 ± 0.0	0.167 ± 0.002	0.323 ± 0.02	0.657 ± 0.0
PMT + CP_1	51.7 ±0.05	2.0 ± 0.2	1.5 ± 0.0	0.231 ± 0.018	0.453 ± 0.009	0.475 ± 0.03
PMU + CP_1	53.5 ± 0.11	8.1 ± 0.5	2.2 ± 0.1	0.123 ± 0.02	0.213 ± 0.02	1.1 ± 0.01

The dissimilar alphabets (a, b, c & d) in the superscripts denote that the mean values are statistically different across columns, the treatment conditions at $p < 0.05$.
Abbreviations: PMU, pin-milled untempered grain; PMT, pin-milled tempered grain; PMT + CP_1, pin-milled tempered grain +25kV/8min cold plasma treatment; PMU + CP_1, pin-milled untempered grain + 25kV/8min cold plasma treatment.

5.3.5. Response surface models for various attributes of cold plasma-treated tempered and untempered PMF bread

The cold plasma-treated tempered and untempered flour has been taken for the future optimization of the bread formulations. The impact of the various formulations on the tempered PMF bread's hardness, specific volume (SV) of the loaf, and Total color change of crust (TCC) was detailed in Table 5.4. The different combinations of the breads showed the hardness values ranging in 5.8 to

14.68 N. The specific volume and crust color change values (ΔE^*) are 2.2 to 3.25 $mL.g^{-1}$ and 3.88 to 12.21, respectively. The objective is that the model remains unaliased where added terms are significant. The model is a good fit as it passes the lack-of-fit test and aims to have the highest adjusted R^2 value (0.885,0.817, 0.872 for hardness, TCC crust, and SV, respectively, for the special cubic model). The special cubic model has an insignificant lack of fit, and it was not aliased, making it the most suitable model compared to the quadratic, linear, special cubic and cubic models. The nonsignificant lack of fit refers to a negligible pure error within the system. This makes the model reliable. The higher the F -values (16.19, 6.71, 4.10 for the hardness, TCC crust, and SV, respectively) for the model indicate the minimal impact of the noise on the model fitting process.

The impact of the various formulations of the untempered PMF bread's hardness, specific volume (SV) of the loaf, and changes in the crust colour was detailed in Table 5.6. The optimal mixture design has different combinations of breads, with the hardness values ranging from 4.53 to 13.14 N. The specific volume and crust color change values (ΔE^*) range from 2.35 to 3.95 $mL.g^{-1}$and 4.49 to 13.97, respectively. The special cubic model has an insignificant lack of fit, and it was not aliased, making it the most suitable model compared to the quadratic, linear, special cubic, and cubic models, like the tempered cold plasma-treated flour. The highest adjusted R^2 value for the special cubic model is 0.928, 0.992,0.994 for hardness, TCC crust, and specific volume, respectively. This model has the highest F- value (8.31, 1.06, 5.97 for hardness, TCC crust, and SV, respectively), showing the minimal impact of the noise in the fitting of the model. The ANOVA data and coefficients for both models are mentioned in Table 5.5& 5.7. Figures 5.2 & 5.3 show the Contour plots describing the effect of the PMT + CP_1 and PMU + CP_1 on various responses at different combinations of refined wheat flour, PMF, and shortening.

5.3.5.1. Hardness

5.3.5.1.1. Tempered PMF bread - The hardness of the bread varied based on the different combinations of the flour. The lowest hardness achieved is 5.8 N at a mix of 86.1 g refined wheat flour, 10.2 g PMF, and 3.6 g shortening. The highest hardness is 14.68 N at a mix of 70 g refined wheat flour, 24.4 g PMF, and 5.5 g shortening agent. Hardness exhibits the negative coefficient (-1.24) with the combination of the refined wheat flour and PMF. Increase the PMF flour in the mix; it increases the hardness of the bread. It is due to the lack of gluten protein, reducing the extensibility and gas-holding capacity, resulting in a denser crumb with higher firmness. PMF also increases the dietary fiber content in the mix, which enhances the stiffness of the cell wall surrounding the gas bubbles, thereby limiting expansion during baking (Marinho et al., 2025). The interaction between the cubic term and the remaining square terms is not significant, such as the interaction between the PMF and shortening, Refined wheat flour, and shortening. The contour plate is the concave downward response surface indicating the non-linear interaction between the independent variables with respect to the hardness. Ranasalva et al. (2014) found that the higher incorporation of fermented pearl millet flour (25%) into the refined wheat flour increases the hardness (21.12 N) of the bread. Jyothi et al. (2020) found that increasing the hardness of the bread through incorporating 10-30% of millet flour into the wheat flour.

5.3.5.1.2. Untempered PMF bread - The lowest hardness is 5.84 N, achieved at the mix of 80 g of refined wheat flour,13.9 g PMF, and 6.0 g of the shortening agent. The highest hardness is 3.14 N at the mix of 73.9 g refined wheat flour,23 g PMF, and 3.0 g of the shortening agent. Cold plasma-treated untempered ($PMU + CP_1$) flour exhibits a positive interaction (15.27) with the refined wheat flour. Not like the tempered cold plasma-treated flour. The contour plot is showing the convex and upward trend of the response surface with the positive influence. $PMU + CP_1$ exhibits

the greatest viscosity profile with the highest peak and final viscosity among the remaining samples. Cold plasma induces changes in the structural and functional properties of the PMF. Partial oxidation enhanced the inter-granular bonding, water absorption capacity by increasing hydrophilicity of the flour through introducing polar functional groups, surface etching of starch granules, and enhanced starch gelatinization, which helps to interact synergistically with the gluten proteins. Naraharasetti et al. (2025b) & Sarkar et al. (2023) found that cold plasma treatment enhanced the functional properties of the PMF, such as the water absorption capacity, oil absorption capacity, and emulsification capacity. Overall, the interaction of the cubic term, such as the refined wheat flour, PMF, and shortening agent the negative interaction (-200.50) in terms of the hardness. Reducing the gluten protein and using higher shortening agent increases the hardness of the bread. The remaining all square terms are not significant in the model.

5.3.5.2. Total color change (ΔE^*) of crust

5.3.5.2.1. Tempered PMF bread - The color changes varied across all combinations. The highest color change was 14.68, seen in the mix at 70 g refined wheat flour, 24.4 g PMF, and 5.5 g shortening agent. The lowest color difference was 5.8 at a mix of 86.1 g refined wheat flour, 10.2 g PMF, and 3.6 g shortening. The refined wheat flour and the PMF showing the antagonist interaction in terms of the total color change of the crust, exhibiting a negative coefficient (-12.74) with the combination of PMF and refined wheat flour. The remaining square and cubic interactions did not show any significant interaction. The contour plot has a concave upward trend for the TCC crust. Pearl millet flour pericarp, aleurone layer naturally contains the phenolic pigment and polyphenolic compounds, making the flour inherently darker (Pawase et al., 2019). The higher the substitution of the refined wheat flour with PMF, enhances the darkness of the bread due to having the hinger content of the phenolic pigment and polyphenolic compounds. It leads to a greater color

difference. Ranasalva et al. (2014) found that incorporating the fermented pearl millet flour into the refined wheat flour shifted the *L** value from white to grey, *a** value from green to red, and *b** values shifted from blue to yellow.

5.3.5.2.2. Untempered PMF bread - The highest color change of crust is 13.97 was obtained at a mix of 76.3 g refined wheat flour,19.1 g PMF, and 4.4 g of the shortening agent. The lowest color change was 4.49 at a mix of 70 g refined wheat flour,26.5 g PMF, and 3.4 g of the shortening agent. The PMF and refined wheat flour showed a positive coefficient (41.02) in terms of the total color change, unlike the tempered cold plasma-treated flour. The contour plot of the TCC crust change is showing the concave upward trend. Similar to the hardness, the cubic term shows a negative relation (-477.40) with the TCC Crust. The remaining square terms are not significant. The higher the addition of the pearl millet flour, will increase the total color change due to the difference in the color of the flour. However, bread made with tempered flour has a high impact on the total color change. It is due to the tempering process that will change the color profile of the flour through moisture migration around the kernel, allowing the cleaner separation of endosperm during milling and minimizing the pigment migration (Fowler, 2013). Zhang et al. (2025b) found that flour obtained from the tempered wheat grain has a higher whiteness value than the normal wheat flour. Tyl et al. (2019) found that tempered krenza grain have the lower *a** value and higher *L*, b** values lead the flour have the lighter ad less reddish/brown shade than the normal flour. Future analysis is required to determine how the tempering process affects the total color change of the bread.

5.3.5.3. Specific volume

5.3.5.3.1. Tempered PMF bread -The highest specific volume of 3.25 $mL.g^{-1}$ was obtained at a mix of 80 g refined wheat flour,13.9 g PMF, and 6.0 g of the shortening agent. The lowest specific

volume of 2.2 $mL.g^{-1}$ was obtained at the mix of 76.3 g refined wheat flour, 19.1 g PMF, and 4.4 g of the shortening agent (Table 5.4). The interaction between the refined wheat flour and shortening shows an antagonistic effect with a negative coefficient (-57.40). In a similar way, the interaction between the PMF and shortening also shows the negative coefficient (-59.80). The interaction of the refined wheat flour and PMF, the interaction of the refined wheat flour, PMF, and Shortening did not show any significance. A higher amount of shortening agents coats the flour proteins and starch granules, limiting the water access and weakening the gluten network formation, which reduces the dough extensibility and gas retention during baking (Pareyt et al., 2011). It leads to a reduction in the specific volume of the bread. Mancebo et al. (2017) found a similar trend in the bread made with rice flour. Dexter et al. (1990) found that specific volume decreased while increasing the shortening agent to an excess level in the wheat bread.

5.3.5.3.2. Untempered PMF bread -The highest specific volume obtained was 3.95 $mL.g^{-1}$ at the mix of 80 g refined wheat flour,13.9 g PMF, and 6.0 g of the shortening agent. The lowest specific volume was the 2.35 $mL.g^{-1}$ obtained at the mix of 73 g of refined wheat flour,20.9 g of the PMF, and 6.0 g of the shortening agent. The interaction between the refined wheat flour and PMF shows the negative coefficient (-0.1320) in terms of the specific volume. The interaction between all remaining terms is not significant. The contour plot of specific volume indicates the concave shape with a downward trend. Substituting the refined wheat flour with PMF leads to a reduction in the specific volume of the bread. PMF reduces the total gluten proteins, weakening the viscoelastic matrix needed to trap the gas from yeast fermentation, resulting in the lower loaf height (Nehra et al., 2021). Maktouf et al. (2016) found that the specific volume of the bread decreases when adding more PMF to the baking mix.

Table 5.4: Independent variables with formulation based on the D-optimal mixture design and responses from the tempered cold plasma-treated PMF bread

Sample	Mixture variables			Response Variables		
	refined wheat flour (X_1, g)	PMF (X_2, g)	Shortening (X_3, g)	Hardness (Y_1, N)	TCC crust (Y_2, -)	SV (Y_3, $mL.g^{-1}$)
S1	76.3	19.1	4.4	9.3 ± 0.1^{cde}	7.25 ± 0.05^{e}	2.30 ± 0.01^{g}
S2	83.6	13.3	3.0	7.19 ± 0.3^{de}	6.71 ± 0.03^{f}	2.66 ± 0.01^{c}
S3	86.1	10.2	3.6	5.81 ± 0.6^{e}	5.9 ± 0.01^{g}	2.59 ± 0.03^{d}
S4	84.4	10.0	5.5	6.96 ± 1.1^{de}	8.28 ± 0.1^{d}	2.37 ± 0.01^{f}
S5	80.0	13.9	6.0	6.38 ± 0.9^{de}	4.74 ± 0.09^{i}	3.25 ± 0.01^{a}
S6	70.0	24.4	5.5	14.68 ± 2.3^{a}	10.47 ± 0.2^{b}	2.32 ± 0.05^{fg}
S7	86.1	10.2	3.6	5.8 ± 0.6^{de}	5.3 ± 0.01^{h}	2.6 ± 0.03^{cd}
S8	76.3	19.1	4.4	9.4 ± 0.1^{bcde}	7.3 ± 0.05^{e}	2.2 ± 0.01^{h}
S9	77.2	16.7	6.0	9.61 ± 0.2^{bcde}	7.16 ± 0.08^{e}	2.46 ± 0.01^{e}
S10	76.3	19.1	4.4	9.63 ± 0.1^{bcde}	6.72 ± 0.04^{f}	2.29 ± 0.01^{g}
S11	80.6	16.3	3.0	8.73 ± 2.5^{cde}	5.8 ± 0.02^{g}	2.95 ± 0.03^{b}
S12	70.0	26.5	3.4	12.71 ± 1.9^{abc}	12.21 ± 0.3^{a}	2.33 ± 0.02^{fg}
S13	73.9	23.0	3.0	13.56 ± 0.8^{ab}	9.72 ± 0.09^{c}	2.98 ± 0.01^{b}
S14	78.0	18.9	3.0	7.08 ± 3.1^{de}	3.88 ± 0.4^{j}	2.48 ± 0.01^{e}
S15	84.4	10.0	5.5	6.7 ± 1.5^{de}	8.1 ± 0.1^{d}	2.2 ± 0.02^{h}
S16	73.0	20.9	6.0	10.16 ± 1.4^{bcd}	10.37 ± 0.07^{b}	2.12 ± 0.02^{i}
S17	76.3	19.1	4.4	9.3 ± 0.1^{cde}	7.2 ± 0.04^{e}	2.2 ± 0.01^{h}

PMF, pearl millet flour; TCC, total color change; SV, specific loaf volume. The dissimilar alphabets (a, b & c) in the superscripts denote that the mean values are statistically different across the columns at $p < 0.05$.

Table 5.5: Coefficient of all terms in L- pseudo coded values for the special cubic model and corresponding ANOVA data describing the effect of varying compositional parameters in the tempered cold plasma-treated PMF bread

Model Terms	Co-efficient (±CI)		
	Hardness (Y_1, N)	TCC crust (Y_2, -)	SV (Y_3, $mL.g^{-1}$)
X_1-Refined wheat flour	5.45 ± 1.31	5.91 ± 1.45	2.85 ± 0.308
X_2 - PMF	13.54 ± 1.45	12.53 ± 1.61	2.78 ± 0.3431
X_3 - Shortening	13.46 ± 81.85	24.43 ± 90.54	48.72 ± 19.34
$X_1 \times X_2$	-1.24 ± 5.62	-12.74 ± 6.22	-0.287 ± 1.33**
$X_1 \times X_3$	0.5655 ± 101.21**	-9.29 ± 111.96**	-57.40 ± 23.91
$X_2 \times X_3$	4.53 ± 101.27**	-21.99 ± 112.03**	-59.80 ± 23.93
$X_1 \times X_2 \times X_3$	-63.67 ± 58.97**	-14.14 ± 65.23**	10.23 ± 13.93**
ANOVA for the model			
P_{lof}	0.315	0.106	0.256
P_{model}	<0.0001	<0.0001	<0.0001
F-value	16.19	6.71	4.10
R^2	0.985	0.960	0.982
Adjusted R^2	0.885	0.817	0.872

PMF, Pearl millet flour; TCC, total color change; SV, specific volume; CI, confidence interval at 95%; lof, lack of fit; X_1, PMF, X_2, and X_3 are the dimensionless L- pseudo coded form of the Refine wheat flour, PMF, and shortening, respectively. All the terms are significant at $p < 0.05$, otherwise mentioned as *(0.05 < p <0.1) or ** (p > 0.1).

Table 5.6: Independent variables with formulation based on the optimal Mixture design and responses from the untempered cold plasma-treated PMF bread

Sample	Mixture variables			Response Variables		
	Refined wheat flour (X_1, g)	PMF (X_2, g)	Shortening (X_3, g)	Hardness (Y_1, N)	TCC crust (Y_2, -)	SV (Y_3, $mL.g^{-1}$)
S1	76.3	19.1	4.4	8.8 ± 0.5^{bcd}	13.97 ± 0.3^{b}	2.88 ± 0.02^{cd}
S2	83.6	13.3	3.0	8.37 ± 0.2^{bcd}	9.21 ± 0.1^{e}	2.9 ± 0.02^{b}
S3	86.1	10.2	3.6	4.53 ± 0.6^{e}	6.72 ± 0.2^{f}	2.83 ± 0.02^{cd}
S4	84.4	10.0	5.5	8.68 ± 0.8^{bcd}	10.07 ± 0.09^{d}	3.14 ± 0.04^{b}
S5	80.0	13.9	6.0	5.84 ± 1.2^{de}	8.82 ± 0.2^{e}	3.95 ± 0.01^{a}
S6	70.0	24.4	5.5	11.75 ± 3.1^{ab}	19.1 ± 0.4^{a}	2.71 ± 0.01^{ef}
S7	86.1	10.2	3.6	4.55 ± 0.7^{e}	6.7 ± 0.1^{f}	2.79 ± 0.03^{de}
S8	76.3	19.1	4.4	8.6 ± 0.4^{bcd}	13.92 ± 0.3^{b}	2.8 ± 0.02^{de}
S9	77.2	16.7	6.0	8.67 ± 0.7^{bcd}	6.77 ± 0.05^{f}	2.85 ± 0.02^{cd}
S10	76.3	19.1	4.4	7.59 ± 0.5^{cde}	4.87 ± 0.2^{g}	2.67 ± 0.02^{fg}
S11	80.6	16.3	3.0	11.55 ± 2.2^{ab}	6.69 ± 0.2^{f}	2.47 ± 0.03^{h}
S12	70.0	26.5	3.4	9.52 ± 1.7^{abc}	4.49 ± 0.08^{g}	2.59 ± 0.05^{g}
S13	73.9	23.0	3.0	13.14 ± 0.9^{a}	12.11 ± 0.1^{c}	2.72 ± 0.06^{ef}
S14	78.0	18.9	3.0	6.97 ± 0.5^{cde}	13.68 ± 0.3^{b}	2.88 ± 0.05^{cd}
S15	84.4	10.0	5.5	8.6 ± 0.7^{bcd}	10.09 ± 0.1^{d}	3.11 ± 0.01^{b}
S16	73.0	20.9	6.0	7.41 ± 0.9^{cde}	5.04 ± 0.07^{g}	2.35 ± 0.03^{i}
S17	76.3	19.1	4.4	8.7 ± 0.5^{bcd}	13.95 ± 0.3^{b}	2.85 ± 0.02^{cd}

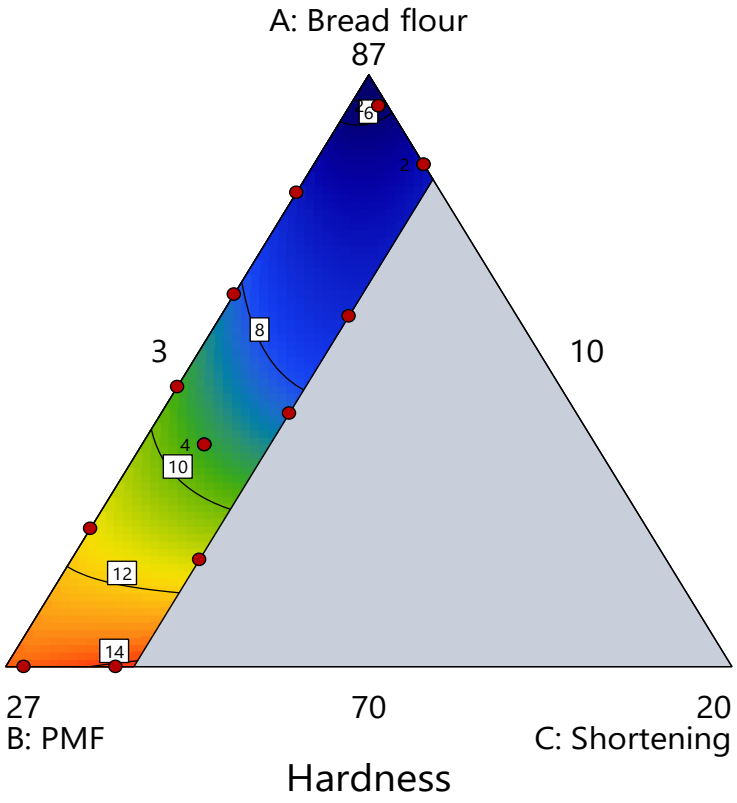
PMF, Pearl millet flour; TCC, total color change; SV, specific volume. The dissimilar alphabets (a, b & c) in the superscripts denote that the mean values are statistically different across the columns at $p < 0.05$.

Table 5.7: Coefficient of all terms in L- pseudo coded values for the special cubic model and corresponding ANOVA data describing the effect of varying compositional parameters in the untempered cold plasma-treated PMF bread

Model Terms	Co-efficient (±CI)		
	Hardness (Y_1, N)	TCC crust (Y_2, -)	SV (Y_3, $mL.g^{-1}$)
X_1-Refinedwheat flour	3.74 ± 1.89	1.03 ± 3.95	2.71 ± 0.328
X_2 - PMF	10.0 ± 2.10	2.61 ± 4.39	2.75 ± 0.365
X_3 - Shortening	51.98 ± 118.27	-365.67 ± 247.27	14.65 ± 20.600
$X_1 \times X_2$	15.27 ± 8.12	41.02 ± 16.98	-0.1320 ± 1.41
$X_1 \times X_3$	-20.6 ± 146.24**	510.14 ± 305.77**	-9.81 ± 25.47**
$X_2 \times X_3$	-40.40 ± 146.33**	538.36 ± 305.95**	-16.39 ± 25.49**
$X_1 \times X_2 \times X_3$	-200.50 ± 85.20	-477.40 ± 178.13	7.73 ± 14.84**
ANOVA for the model			
P_{lof}	0.3712	0.2396	0.2357
P_{model}	<0.0001	<0.0001	<0.0001
F -value	8.31	1.06	5.97
R^2	0.9286	0.9922	0.9945
Adjusted R^2	0.8058	0.8875	0.8912

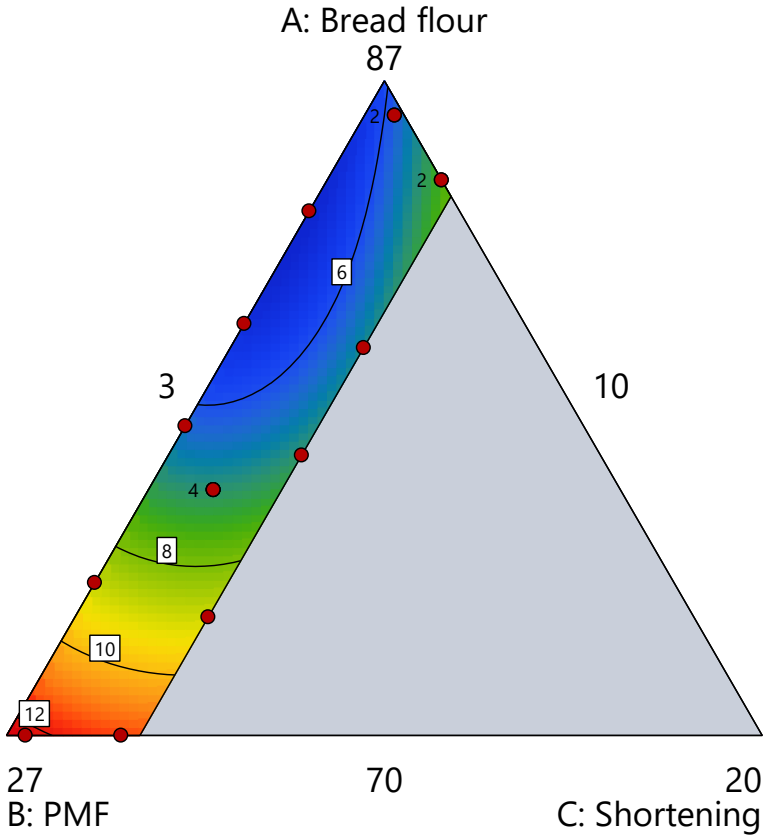
PMF, Pearl millet flour; TCC, total color change; SV, specific volume; CI, confidence interval at 95%; lof, lack of fit; X_1, PMF, X_2, and X_3 are the dimensionless L- pseudo coded form of the Refines wheat flour, PMF, and shortening, respectively. All the terms are significant at $p < 0.05$, otherwise mentioned as *(0.05 < p <0.1) or ** (p > 0.1).

a)



Hardness

b)



TCC crust

c)

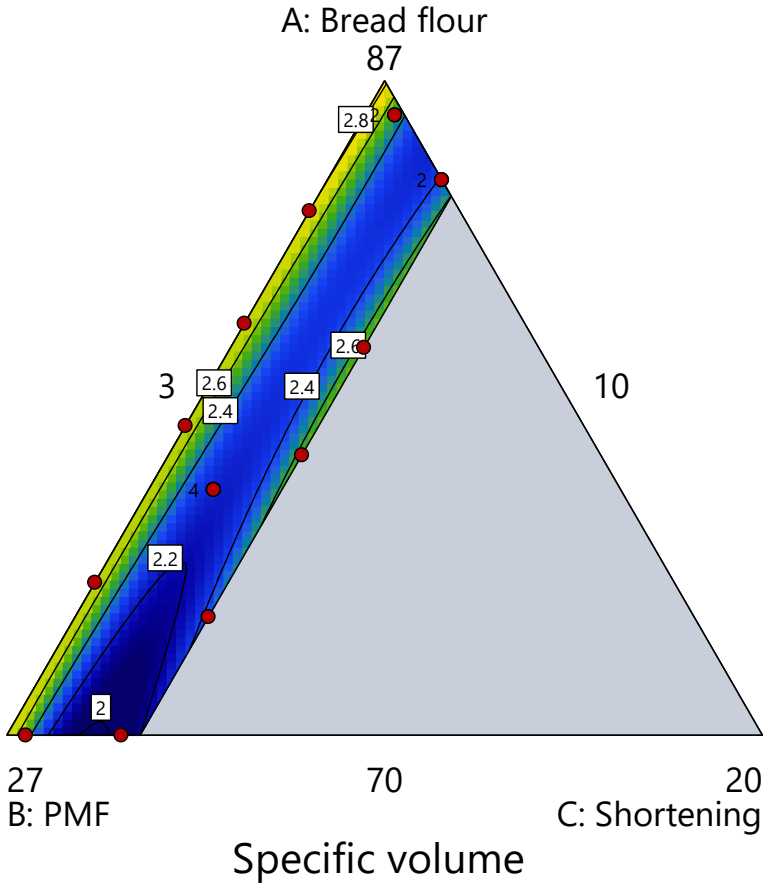
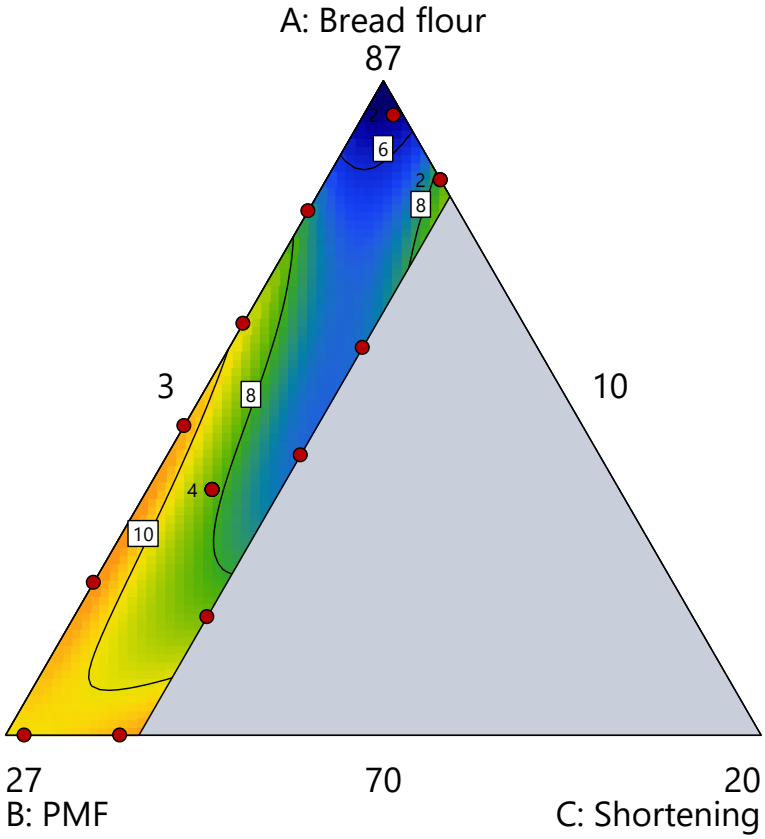


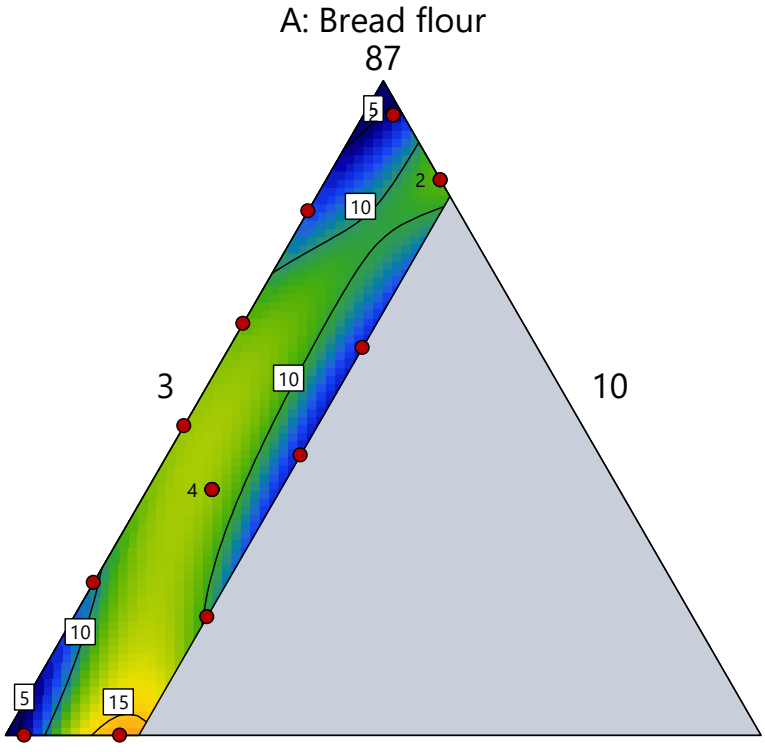
Figure 5.2: Contour plots describing the effect of the PMT + CP_1 on various responses at different combinations of Refined wheat flour, PMF, and shortening. a) Hardness (Y_1, N) b) TCC crust (Y2, -) c) SV (Y3, $mL.g^{-1}$).

a)



Hardness

b)



TCC crust

c)

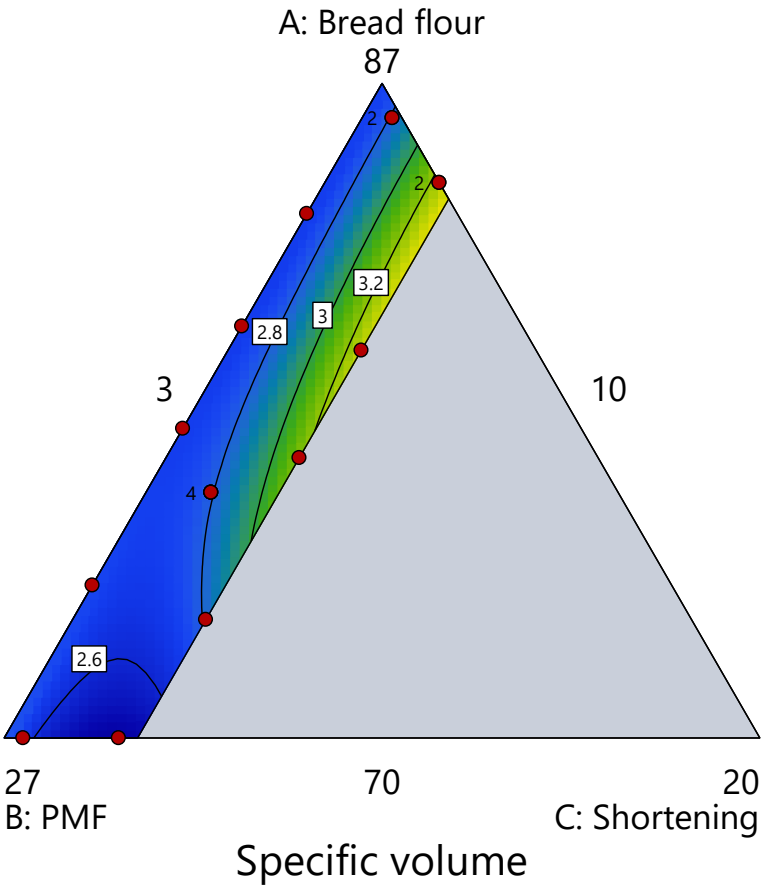


Figure 5.3: Contour plots describing the effect of the PMU + CP_1 on various responses at different combinations of refined wheat flour, PMF, and shortening. a) Hardness (Y_1, N), b) TCC crust (Y2, -), and c) SV (Y3, $mL.g^{-1}$).

5.3.6. Optimization of the baking mix

The criteria for optimizing the formulation of bread are mentioned in Table 5.8. The objective of the optimization in the formulation is to maximize the replacement of the refined wheat flour with pearl millet flour. The shortening agent is kept in the range of 3-6 g in the bread formulations. The objective aims to minimize the bread hardness, maximize the specific volume, and keep the total color change of the crust in a range. Numerical optimization was done by using the higher relative importance to hardness (5) followed by the SV (4) and PMF (4). All the remaining parameters

have the default values, such as the wheat flour (3), shortening (3), and TCC crust (3). The higher importance of the specific volume and hardness is due to the good quality of the bread, which is determined based on the higher specific volume and lower hardness (Stefan et al.,2024). Relative importance helps to achieve the desired properties in the end product. Numerical optimization suggests that the best formulation for the tempered pearl millet flour (PMT + CP_1) with Dov =0.559, having the mixture of 85.5% refined wheat flour,11.5% PMF, and 3 g of the shortening agent. The bread made with the optimization formulation has the hardness, specific volume and TCC crust of 6.06 N, 2.82 $mL.g^{-1}$, and 5.47, respectively. The optimized condition for the untempered pearl millet flour (PMU + CP_1) with D_{ov}=0.657, having the mixture of the formulation is 75.7 g refined wheat flour, 18.3 g PMF, and 6 g of the shortening agent, with the hardness, specific volume, and TCC crust is 7.32 N,3.04 $mL.g^{-1}$, and 6.60, respectively. The actual values close to the predicted values with a < 4 % deviation (Table 5.8) prove the validity of the model. Figure 5.4 shows the images of the C-cell analysis for both optimized conditions and 100% refined wheat flour. Further baking properties were analysed for the optimized conditions with respect to the bread made with the 100% refined wheat flour (Table 5.9). There is a significant difference in the hardness of the bread for the optimized conditions PMT + CP_1 (5.95 N), PMU + CP_1 (7.15 N), with respect to the 100% refined wheat flour (2.094N). Cohesion (-), springiness (%), gumminess (N), and chewiness (N) for the optimized conditions, such as PMT + CP_1 (0.29, 87%, 1.75N, 1.49N), PMU + CP_1 (0.28, 89%, 2.02N, 1.79N), did not show any difference with respect to the optimized conditions. However, there is a significant difference with respect to the 100 % refined wheat flour (0.423, 97.2%, 0.885 N, 0.86N). Similarly, C-cell analysis, except for the wall thickness (mm) remaining parameter, such as the slice area (mm^2), number of cells (-), cell diameter (mm), and area of cells (%), of the PMT + CP_1 (3699 mm^2, 2066, 2.3 mm, 53.6%), and

PMU + CP_1 (4103 mm^2, 2152, 2.5mm, 56.23%) shows the significant difference with respect to the bread made of 100% refined wheat flour (5334 mm^2, 2752, 3.221 mm, 58.37 %). The wall thickness of bread is 0.457 mm,0.44 mm, and 0.45 mm for the PMT + $CP_{1,}$ PMU + $CP_{1,}$ and refined bread flour, respectively. All the texture aspects and C- cell analysis profile were significant for both optimized conditions except the hardness. The quality of the bread is attributed to the good loaf volume with lower hardness (Pulivarthi et al., 2022). Ranasalva et al. (2014) found that the addition of fermented pearl millet flour to the bread mix at 10%, 15%, and 20% to the mix resulted in specific volumes of 5.61 mL. g^{-1} , 5.55 mL. g^{-1} , and 5. 47mL.g^{-1} respectively, and hardness of 12.22 N, 16.7N, and 19.65 N, respectively. Nehra et al. (2021) observed that the addition of the PMF to the wheat mix with the 10%, 20%, and 30% substitution gave the bread with the specific volumes of 3.02 mL.g^{-1}, 2.75 mL.g^{-1}, and 2.59 mL.$g^{-1,}$ respectively. Maktouf et al. (2016) found that bread made with substituting the wheat flour with the 5% and 10% PMF has the 121.12 N, 124.11 N hardness,4.30 mL.g^{-1}, 2.80 mL.g^{-1} specific volume,0.36, 0.41 cohesion,5.64 mm, 6.13mm springiness, 129.51 N · mm, 131.45 N · mm chewiness, respectively. Bread made with the cold plasma-treated pearl millet exhibits better textural properties than bread made with the untreated pearl millet flour, showing the positive effect on the flour's functional properties, enhancing the viscoelastic and dough properties. So far, no study has been conducted on analysing the bread textural properties with the cold plasma-treated PMF. However, some studies are conducted on other grains. Chaple et al. (2023) found that coldplasma treatment of wheat flour have enhance the texture profile analysis of the bread, which positively affects the expansion ratio, crust color, dough stability, and dough development time. Menkovska et al. (2014) found that cold plasma treatment of the wheat flour demonstrated the expansion of the specific volume, appreance and porosity structure along with the dough stability.

Table 5.8: The set of various parameters for the optimization of tempered and untempered cold plasma-treated PMF bread

Parameters	Goal	Lower limit		Upper limit		Relative importance	Optimized condition		Actual value	
		PMU + CP_1	PMT + CP_1	PMU + CP_1	PMT + CP_1		PMU + CP_1 (D_{ov}=0.657)	PMT + CP_1 (D_{ov}=0.559)	PMU + CP_1	PMT + CP_1
Refined wheat flour (X1,g)	minimize	70	70	87	87	3	75.67	85.50	75.67	85.50
PMF (X_2, g)	maximize	10	10	27	27	4	18.32	11.49	18.32	11.49
Shortening (X_3, g)	in range	3	3	6	6	3	6	3	6	3
Hardness (Y_1, N)	minimize	2.35	2.8	3.95	3.25	5	7.32	6.06	7.15 ± 0.15^a	5.95 ± 0.36^b
TCC crust (Y_2, -)	in range	4.53	5.8	13.14	14.68	3	6.60	5.47	6.71 ± 0.25^a	5.62 ± 0.12^b
SV (Y_3, $mL.g^{-1}$)	maximize	4.49	3.8	19.1	12.21	4	3.04	2.82	3.14 ± 0.13^a	2.75 ± 0.09^b

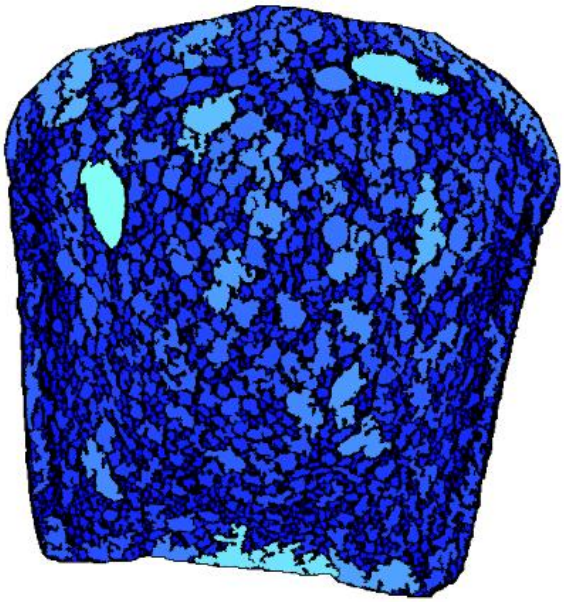
PMF, Pearl millet flour; TCC, total color change; SV, specific volume. The dissimilar alphabets (a, b & c) in the superscripts denote that the mean values are statistically different across the rows at $p < 0.05$.

Table 5.9: The set of baking properties for the optimization of tempered and untempered cold plasma-treated PMF bread

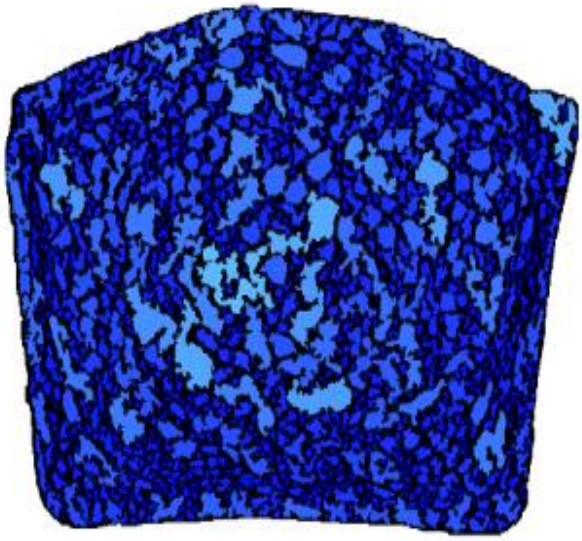
Parameters	100 % Refined wheat flour	PMT + CP_1	PMU + CP_1
Hardness (N)	2.094 ± 0.28^a	5.95 ± 0.36^b	7.15 ± 0.15^c
Cohesion	0.423 ± 0.060^a	0.29 ± 0.02^b	0.28 ± 0.03^b
Springiness (%)	97.2 ± 2.33^a	87 ± 1.107^b	89 ± 2.52^b
Gumminess (N)	0.885 ± 0.205^a	1.72 ± 0.321^b	2.02 ± 0.15^b
Chewniess (N)	0.86 ± 0.205^a	1.49 ± 0.125^{ab}	1.79 ± 0.415^b
Slice area (mm^2)	5334 ± 402.8^a	3699 ± 319.0^b	4103 ± 244.5^b
Wall thickness (mm)	0.457 ± 0.007^a	0.44 ± 0.0033^a	0.45 ± 0.014^a
Number of cells	2752 ± 358.4^a	2066 ± 152.5^b	2152 ± 127.2^b
Cell diameter (mm)	3.221 ± 0.221^a	2.3 ± 0.1755^b	2.5 ± 0.405^b
Area cells (%)	58.37 ± 0.928^a	53.6 ± 0.77^b	56.23 ± 0.731^c

The dissimilar alphabets (a, b, c & d) in the superscripts denote that the mean values are statistically different across the treatment conditions at $p < 0.05$.

a)



b)



c)

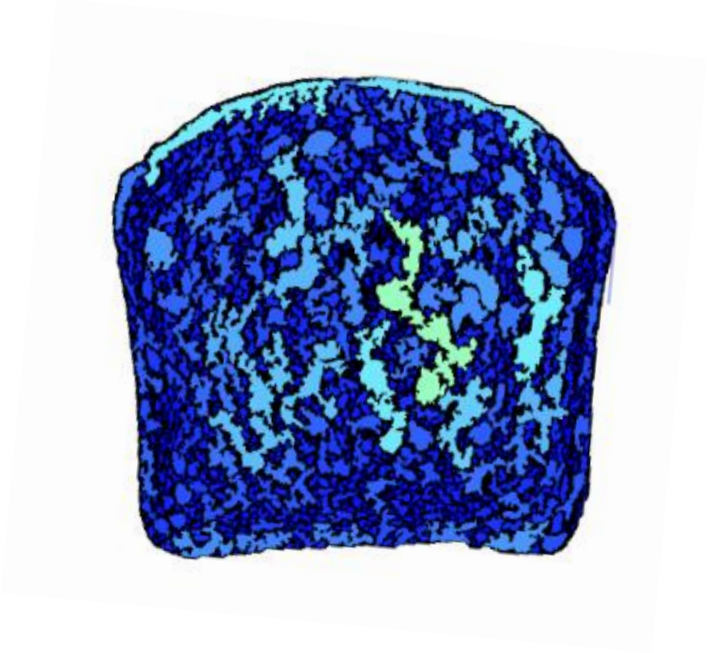


Figure 5.4: The C – cell images of the slices bread with optimized conditions, a)100% refined wheat flour, b) tempered cold plasma-treated pearl millet flour, b) untempered cold plasma-treated pearl millet flour

5.4. Conclusion

Incorporating the cold plasma-treated pearl millet flour into bread-making is a novel approach to improve textural attributes. Pearl millet grain milled with the pin mill and tempered condition has achieved the highest milling yield of 93.5% than the hammer-milled and untempered flour. The tempering process has enhanced the yield in both milling process such as the hammer mill and pin mill. Pin-milled flour had a uniform particle distribution exhibiting a unimodal distribution with the greater yield to extract to the flour in both tempered and untempered conditions, with 93.5% and 91.5%, respectively. Through the cold plasma treatment of the pin-milled pearl millet flour, enhance the viscoelastic properties of the flour. An optimized blend for making bread with the tempered flour composed of 85.5 g wheat flour,11.5 g PMF, and 3 g of shortening, having the hardness, total color change of crust, and specific volume of 6.06 N, 5.47, and 2.82 $mL.g^{-1,}$

respectively. Similarly, optimized blend for making bread with the untempered pearl millet flour has 75.67 % wheat flour, 18.32% PMF, and 6 g of the shortening agent, having the hardness, Total color change of crust, and specific volume are 7.32 N,6.60, and 3.04 $mL.g^{-1}$ respectively. Except for the hardness, the other baking properties, such as the texture profile analysis and C-cell analysis, did not show any significant difference between the optimized conditions, such as the pin - milled cold plasma-treated tempered and untempered flour. Overall, cold plasma treatment helps to enhance the textural properties of the bread by improving the viscoelastic properties of the gluten-free flour. Substitution of the refined wheat flour with PMF leads to a reduction in the specific volume and collapsing the bread while baking. Future research should focus on the sensory analysis and impact of the gums, emulsifiers, and dough conditioners on the specific volume and the textural attributes of the optimized conditions of PMF-substituted bread.

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Chapter 6
Summary and Future Work

The first objective aimed to achieve the inactivation of quality-deteriorating enzymes such as lipase, lipoxygenase, polyphenol oxidase (PPO), and peroxidase (POD) by using cold plasma. Cold plasma treatment at 25 kV for 8 min led to 95.9%, 99.2%, 94.8%, and 100% inactivation of lipase, lipoxygenase, peroxidase, and PPO activity, respectively. Cold plasma-induced enzyme inactivation followed nth-order kinetics, such as 1.55, 1.35, 1.15, and 1.05 for lipase, peroxidase, lipoxygenase, and PPO, respectively. A higher reduction of the tannin (77.7 g/hg) and phytic acid (24 g/hg) content was obtained in the cold plasma treated samples (25kV/8min) than in the steam treatment (100 ℃/4). Steam treatment at 100 ℃/4 min inactivated lipase, lipoxygenase, peroxidase, and PPO by 97.9, 98.8, 97.6, and 100 %, respectively. Both treatments reduce the formation of the secondary lipid oxidation products, such as the free fatty acids and TABRS. Cold plasma treatment (25 kV/8 min) enhances the functional properties such as the water absorption capacity (1.56-2.20 g/g), oil absorption capacity (1.43-1.94 g/g), and emulsification capacity (50-54 g/hg). The physicochemical characteristics of the pearl millet flour are not affected by the cold plasma treatment, and it helps to preserve the heat-sensitive nutrients.

The second objective aims to achieve microbial safety in pearl millet flour by reducing the presence of Salmonella through the use of cold plasma exposure. The highest Salmonella inactivation of 3.0 log cycle was achieved with the combination treatment of such as the cold plasma treatment of the grain (28kV/60 min) followed by tempering with plasma-activated water (28kV/60min) and cold plasma treatment of the flour at 28kV/60 min. A+B+C treatment condition

shows a decrease in true, bulk, tapped density, an increase in the geometric mean diameter, and changes in the color profile observed due to the tempering of the grains.

The third objective aims to select the pearl millet grain milling methods using a pin mill and a hammer mill, as well as to determine the dough rheological properties. Furthermore, it aims to develop a functional bread using cold plasma-treated tempered and untempered pearl millet flour (PMF) using the D-optimal mixture design. Pin-milled PMF has a uniform particle distribution, and tempered pearl millet flour exhibited the highest yield, 93.5%. An optimized blend composed of 85.5 g refined wheat flour, 11.5 g PMF, and 3 g of shortening, having the hardness, total color change of crust, and specific volume 6.06 N, 5.47, and 2.82 $mL.g^{-1,}$ respectively. Cold plasma treatment at 25 kV/8 min enhances the overall functional properties and texture attributes of the bread.

Future work

Future work needs to focus on assessing the shelf life and impact on the nutrients. Impact of the gums, emulsifiers, and dough conditioners on the specific volume, textural attributes, and sensory aspects of the optimized conditions of PMF-substituted bread. Additionally, the resistance of *Salmonella* to longer exposure duration at high voltage of the cold plasma treatment, and the efficacy of the cold plasma technology on other pathogens in the pearl millet processing.