



Microbial Growth Kinetics of Fermenting Botanicals Used as Gluten-Free Flour Blends [†]

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Abstract: The fragmentary and whole substitution of wheat flour with flour blends is an alternative approach for producing cheaper, nutrient-rich, and comparatively advantageous gluten-free foods through fermentation. Dry samples of sweet potato, pigeon pea, and maize botanicals were purchased from local vendors, authenticated and processed before spontaneous fermentation at room temperature. The pH and microbiological growth patterns of the fermenting botanicals were evaluated every 12 h for 72 h, using standard test protocols. It revealed that the rates of growth of isolated microorganisms were affected by pH; all the botanicals fermented had a reduction in their pH values. Acids were produced during fermentation, leading to a reduction in pH. Bacteria growth on the fermenting samples on nutrient agar reveals that the bacterial load increased with fermentation time, from 7.52 Log₁₀ CFU/g to 10.6 Log₁₀ CFU/g (sweet potato); 6.3 Log₁₀ CFU/g to 10.54 Log₁₀ CFU/g (pigeon pea), and 6.3 Log₁₀ CFU/g to 10.54 Log₁₀ CFU/g (maize). On MacConkey agar, the bacterial load on all samples started after 24 h of fermentation, peaked at 48 h, and gradually reduced towards 72 h of fermentation. There was increase in fungal growth with time from 0 to 36 h across all samples. The microorganisms isolated can be categorized into lactic acid bacteria, spore formers, Enterobacteriaceae, Staphylococcace, yeast, and molds. Fermentation of botanicals over 72 h results in organic acid formation, which lowers pH; this attribute helps in checkmating undesirable microorganisms capable of affecting the production of gluten-free flours with good keeping qualities.

Keywords: microbial kinetics; food fermentation; botanicals; flour blends



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1. Introduction

Food, whether in liquid or solid form, is essential for maintaining metabolic activities in humans [1,2]. Various food processing methods, such as germination, roasting, boiling, and fermentation, enhance the nutritional quality of foods by reducing anti-nutritional factors like tannins, phytic acid, polyphenols, and enzyme inhibitors [3–5]. However, each method has limitations. For example, while germination improves nutrient bioavailability and enzyme activity, it can be labor-intensive [6,7]. Roasting enhances sensory attributes but may degrade heat-sensitive vitamins [8]. Boiling, though simple, leads to significant nutrient loss, particularly water-soluble vitamins like Vitamin C (up to 50%) and minerals (60–70%) [9].

Fermentation, a well-established food processing method, enhances the digestibility, safety, and shelf life of foods by promoting the growth of beneficial microorganisms [10,11]. It produces natural bio-preservatives that inhibit spoilage microbes while enriching the food with probiotics and essential nutrients [12–14]. Fermentation remains a cornerstone of food preservation, widely used in the production of dairy, beverages, and other fermented foods [15].

Wheat (*Triticum* spp.) is the predominant grain used in flour-based products due to its gluten content, which provides desirable baking properties [4]. However, global wheat shortages, exacerbated by geopolitical conflicts, and increasing cases of gluten intolerance (e.g., celiac disease), necessitate the development of gluten-free alternatives [16–19]. Composite flour blends derived from tubers, cereals, and legumes offer a potential substitute [20,21]. Fermentation plays a crucial role in improving the physicochemical and techno-functional properties of such flours [14].

pH regulation during fermentation is critical, as microbial enzymes require specific pH levels for optimal growth and metabolic activity [22]. The enzymatic breakdown of phytates enhances the bioavailability of proteins and minerals such as iron, zinc, calcium, and magnesium [23]. Additionally, organic acids (e.g., lactic and acetic acids) contribute to flavor development and microbial stability [24,25]. Maintaining a pH of ≤ 4.6 throughout fermentation is essential for food safety, as it inhibits most pathogenic microorganisms [26].

Microorganisms involved in fermentation include lactic acid bacteria (LAB), yeasts (*Saccharomyces cerevisiae*), molds (*Amylomyces rouxii*, *Endomycopsis fibuligera*), and bacteria such as *Zymomonas mobilis* [14,25,27]. These microbes break down complex macromolecules (e.g., starches, lipids, proteins) into simpler, bioavailable components [28]. The production of organic acids and ethanol during fermentation creates a hostile environment for spoilage and pathogenic microorganisms [29].

With increasing consumer demand for affordable, nutrient-rich, and functional foods [30], there is a need to explore underutilized botanical resources for gluten-free flour development [31,32]. This study examines the 12-hourly changes in pH and microbial growth during the spontaneous fermentation of sweet potato, pigeon pea, and maize over 72 h. The findings will help determine their suitability for use in composite gluten-free flour formulations.

2. Materials and Methods

2.1. Sourcing and Preparation of Samples

The methods, as previously reported by [16], were followed in sourcing, authenticating, and preparation of the raw botanicals samples (yellow-fleshed sweet potato cultivar, yellow maize grain variety and pigeon peas).

2.2. Fermentation of Samples

The botanical samples were steeped in water and allowed to spontaneously ferment in a closed system for 72 h at 28 ± 2 °C. Temperature, moisture, and oxygen levels were held constant, following the procedures described by [16].

2.3. Determination of pH Variation with Fermentation Time

A Pye Unicam pH meter (Model PW9409) was used to determine the pH variation of each homogenate 12-hourly during fermentation. Two (2) microliters of each sample was mixed with 100 mL of distilled water and homogenized before the meter was used for pH determination.

2.4. Microbiological Analysis

Microbiological analysis was also carried out 12-hourly on the fermenting botanical samples to determine the total microbial counts for the viable bacteria and fungi as described by [33], and modified by [34].

At the onset of fermentation, 1 mL was aseptically obtained from each sample and introduced into 9 mL of peptone water, after which a chronological 10-fold serial dilution was carried out. An aliquot from each diluted sample was taken and plated in triplicates using the pour plate method into the following agar mediums: nutrient agar (NA), MacConkey agar (MCA) for bacteria, and potato dextrose agar (PDA) for fungi. They were later incubated at 37 °C for 24–48 h and at room temperature (25 ± 2 °C) for bacteria and fungi, respectively. The entire process was repeated 12-hourly for 72 h.

Isolation and Enumeration of Bacteria and Fungi

All colonies with different morphologies were enumerated and expressed as colony forming units per gram (cfu/g) of samples, isolated as pure cultures and stored at 4 °C and at room temperature as agar slants for further characterization. The identification of the various bacterial and fungal species was confirmed, using standard morphological, biochemical, and molecular methods [34–36].

2.5. Determination of Microbial Kinetics Equation

The kinetic equation was determined using the simple regression equation by [37]

$$Y = a + bX + \epsilon$$

where the following definitions hold:

Y is the dependent variable (pH/microbial growth readings);

X is the independent variable (time);

a is the intercept;

b is the slope;

ϵ and is the residual (error).

2.6. Statistical Analysis

The pH and microbiological analysis of all samples were carried out in triplicates and the results are presented as means. Statistical differences between the means were determined by one-way analysis of variance (ANOVA) using IBM SPSS Statistics 24.

3. Results and Discussion

In this study, we investigated the pH dynamics and microbial growth kinetics during the fermentation of sweet potato, maize, and pigeon pea flours over a 72 h period, with evaluations every 12 h. The pH of all fermenting samples showed a consistent decline, indicating acid production during fermentation (Figure 1). Sweet potato had the highest initial pH, dropping from 8.6 to 5.1, followed by maize flour, which declined from 6.5 to 3.4. Pigeon pea had the lowest pH, further decreasing as fermentation progressed. Our findings align with studies on the fermentation of cereal and legume flours. For instance, in ref. [38], during the fermentation of maize-pigeon pea blends, a gradual decrease in pH from 6.52 to 5.34 was observed over a 48 h steeping period, followed by a further decrease to 3.74 after 48 h of souring. Similarly, in maize flour fermentation, the pH declined from 6.30 to 3.89 over 120 h, indicating significant acidification [39].

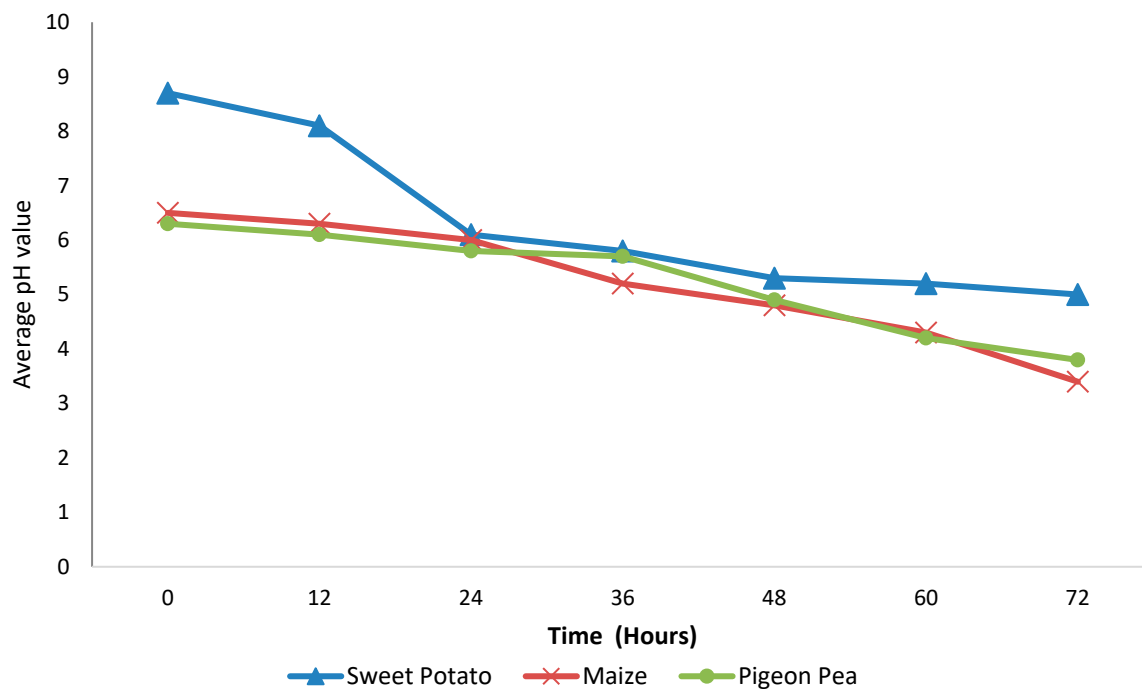


Figure 1. pH of the fermenting botanicals.

The rates of growth of microorganisms isolated from this study were affected by pH relative to time. The initial pH may be suitable, but because of competitive flora or the growth of the organism itself, the pH may become unfavorable. Conversely, the initial pH may be restrictive, but the growth of a limited number of microorganisms (such as the neutrophilic bacteria) may alter the pH to a range that is more favorable for the growth of many other microorganisms [40]. All the samples fermented had a reduction in their pH values. These findings agree with the reports of [39–41] who observed a reduction in pH for fermenting pigeon pea, sweet potato, and maize flours, respectively. However, ref. [39] also observed an increase in pH at the 96th hour of fermentation. During fermentation, the swift formation of organic acids such as lactic acid and the reduction in pH of the medium is a frequent feature, which helps in preventing substandard fermentation by undesirable microbes [41].

Since the time and pH readings are going in opposite directions, this means that the longer the time, the lower the pH readings. This shows that time has a negative effect on the pH readings. The reduction in pH significantly influenced the microbial ecology of the fermenting flours. Lactic acid bacteria (LAB), known for their acid tolerance, thrived under these conditions, leading to an increase in their populations. This proliferation of LAB not only contributes to the desirable characteristics of fermented foods but also suppresses the growth of less acid-tolerant microorganisms, including certain spoilage bacteria and pathogens [38]. The dominance of LAB during fermentation has been linked to improved nutritional profiles and the enhanced safety of the final product.

The kinetic equation specific for this pH process is as follows

$$Y = a - bX + \epsilon$$

where the following definitions hold:

Y is the dependent variable (pH readings);

X is the independent variable (time);

a is the intercept;

b is the slope;

ϵ and is the residual (error).

The heterophilic bacteria growth of the fermenting flour samples on nutrient agar is displayed in Figure 2. It reveals that the bacteria load increased with fermentation time. Bacteria growth in sweet potato increased from 7.52 Log₁₀ CFU/g to 10.6 Log₁₀ CFU/g. Pigeon pea flour bacterial growth increased from 6.3 Log₁₀ CFU/g to 10.54 Log₁₀ CFU/g. Maize flour had a bacteria count from 6.3 Log₁₀ CFU/g to 10.54 Log₁₀ CFU/g.

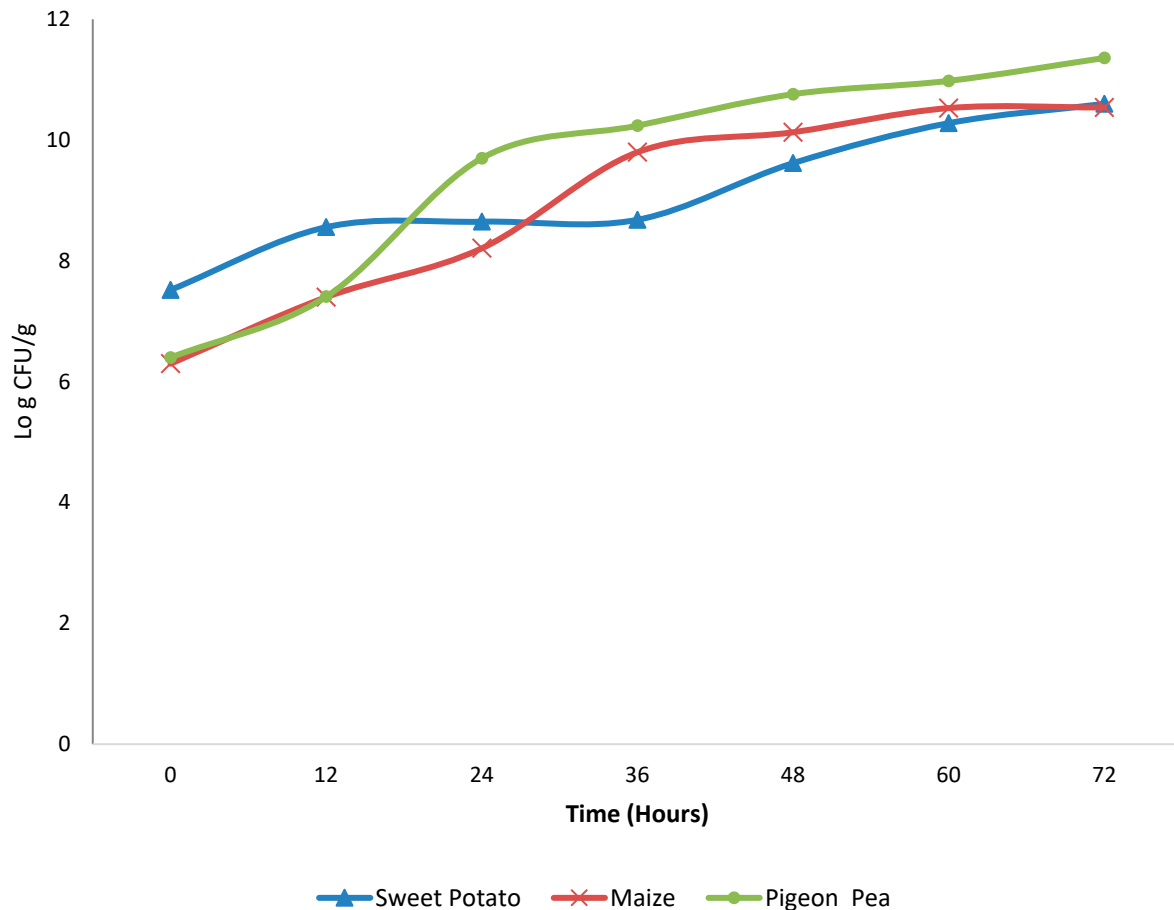


Figure 2. Bacterial growth on nutrient agar.

These findings are in agreement with that of [42,43] who observed an increase in microbial load in the fermentation of tempeh and pigeon peas. However, it differs from that of [44] whose study was on the fermentation of water yam. They explained that the decrease in microbial population may be due to an increase in acidic values (reduced pH values) of the sample as fermentation progressed. The gradual increase in bacterial load on nutrient agar from 0 h to 72 h of fermentation might be due to the increase in the number of lactic acid bacteria (LAB). LAB are dominant in the fermentation of cereals and legumes, as they occur in environments rich in amino acids, vitamins, purines, and pyrimidines contributing specifically to the advancement of flavor in diverse fermented food commodities [45,46]. The presence of a high number of LAB during fermentation suggested that there was interaction with fungi as they may obtain nutrients from mold or yeast metabolism [42].

Bacteria growth on MacConkey agar for the fermenting botanicals is shown in Figure 3. The bacteria load for all samples only started after 24 h of fermentation, peaked at 48 h, and reduced gradually towards 72 h of fermentation. After 24 h, growth in sweet potato ranged between 3.70 Log₁₀ CFU/g (36 h) to 4.40 Log₁₀ CFU/g (48 h). For maize, bacterial growth ranged between 4.16 Log₁₀ CFU/g (60 h) to 8.91 Log₁₀ CFU/g (48 h). Growth on pigeon

pea ranged between 4.57 Log₁₀ CFU/g (60 h) and 5.59 Log₁₀ CFU/g (36 and 48 h). Similar findings were reported by [38,42].

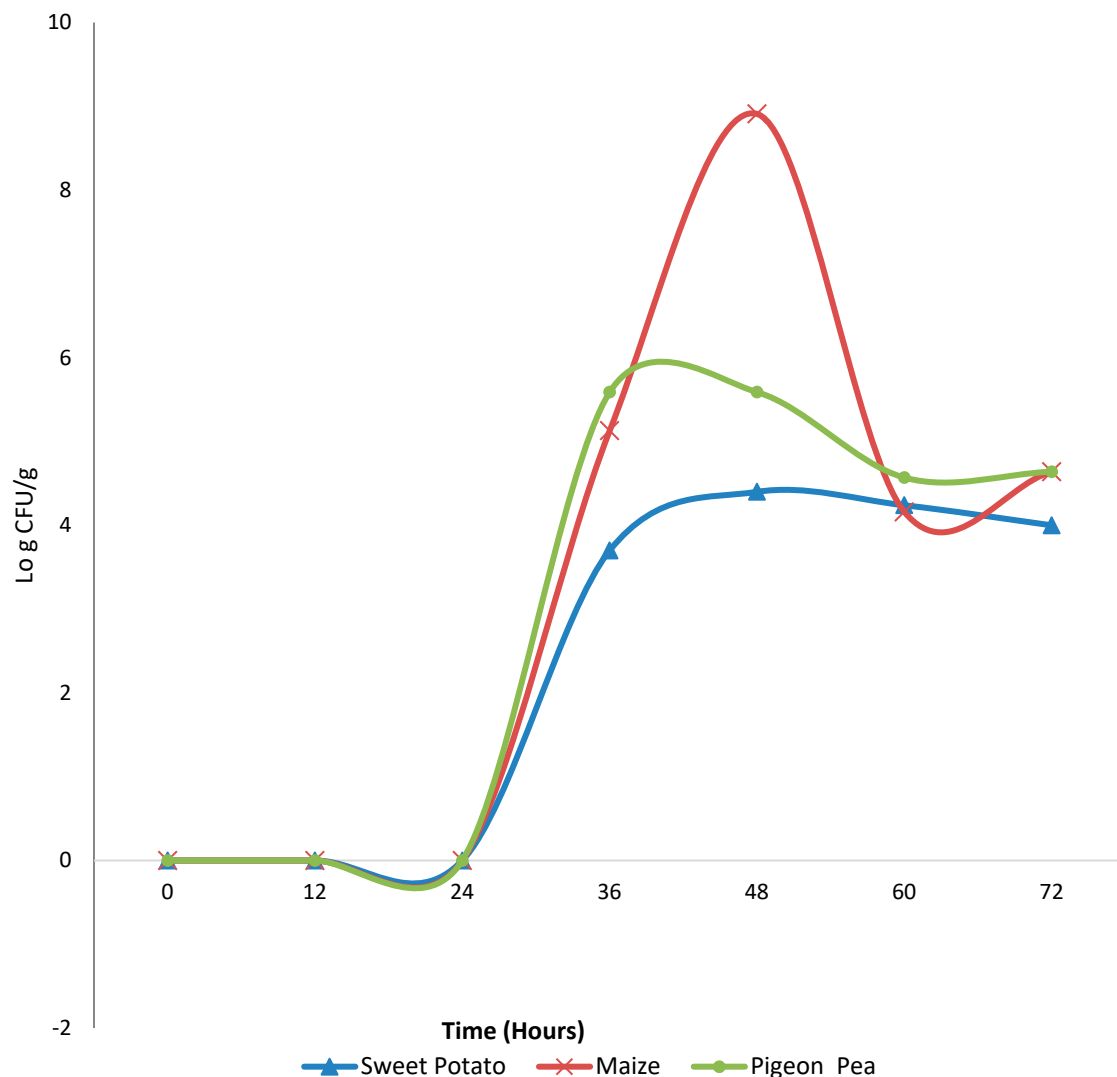


Figure 3. Bacterial growth on MacConkey agar.

Natural fermentation that occurs during soaking has a risk of being overgrown by spoilage bacteria [38,47]. However, their inhibition at the initial 24 h of fermentation time may be due to an increase in the number of desirable bacteria like LAB, whose action affected the growth of undesired bacteria [42].

The fungal growth of the fermenting flour samples (Figure 4) revealed that there was an increase in fungal growth with time from 0 h to 36 h across all samples, but there was no fungal growth seen at 72 h for pigeon pea flour. From 48 h, growth was not as linear as the first 36 h. Fungal growth on sweet potato flour ranged from 1.6 Log₁₀ CFU/g (0 h) to 3.93 Log₁₀ CFU/g (48 h), in maize flour, it was from 1.48 Log₁₀ CFU/g (0 h) to 4.38 Log₁₀ CFU/g (60 h) and for pigeon pea flour, from 0 Log₁₀ CFU/g (72 h) to 3.85 Log₁₀ CFU/g (36 h). Molds play important roles in the fermentation of foods and they are most common in acidic foods [48]. These findings are in line with those of [40,44,49,50].

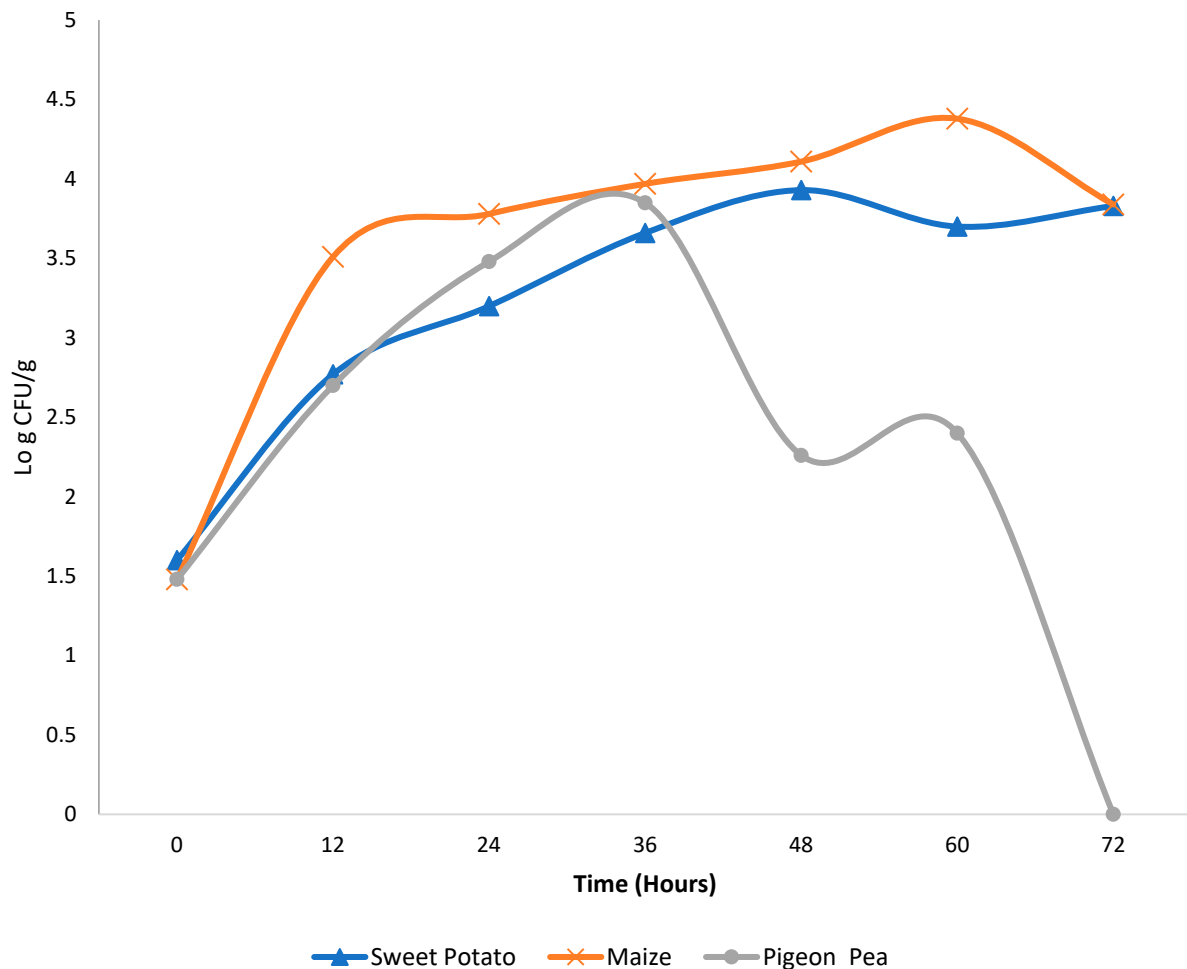


Figure 4. Fungal growth on potato dextrose agar.

From Figures 2–4, it can be seen that the time and microbial load are going in same directions. This shows that time has a positive effect on microbial growth. This means that the longer the time, the higher the microbial growth readings.

The general formula for the time and microbial growth is

$$Y = a + bX + \epsilon$$

where the following definitions hold:

Y is the dependent variable (microbial readings/growth);

X is the independent variable (time);

a is the intercept;

b is the slope;

ϵ and is the residual (error).

The microbial profile of fermented flours in this study can be categorized into lactic acid bacteria, spore formers, enterobacteriaceae, *Staphylococcus*, yeast, and molds, as reported by [35]. Since the sources of these microorganisms could either be from the human skin, cooking utensils, processing equipment, the environment, and water, or from the seeds, there is the need for a microbiological quality control guideline for composite flour blends, which should help distinguish between unacceptable and acceptable fermented foodstuffs because various microbes have been transmitted through food products [37].

4. Conclusions

This study demonstrated that the fermentation of sweet potato, maize, and pigeon pea flours over 72 h resulted in significant biochemical and microbiological changes, with a notable reduction in pH and an increase in microbial load. The pH decrease was primarily due to the production of organic acids, which created an environment favorable for lactic acid bacteria (LAB) while inhibiting the growth of spoilage microorganisms and potential pathogens. This acidification played a crucial role in enhancing food safety, prolonging shelf-life, and improving the sensory attributes of the fermented flours.

Microbial growth dynamics showed that LAB dominated the fermentation process, contributing to the development of desirable functional and probiotic properties. However, the growth trends of bacteria and fungi varied across the different flour types, influenced by pH fluctuations and competitive microbial interactions. The presence of Enterobacteriaceae and other potentially harmful bacteria on MacConkey agar highlights the need for controlled fermentation practices to ensure microbial safety.

The findings from this study support the use of controlled fermentation as an effective method for improving the nutritional value, digestibility, and safety of botanical flours. These results have practical applications in food industries, particularly in developing countries where fermented foods serve as dietary staples. The dominance of LAB suggests potential probiotic applications, while the reduction in pH can be leveraged for natural food preservation. Future research should explore optimizing fermentation conditions, evaluating the bioavailability of nutrients, and assessing the potential of these flours in functional food formulations. Additionally, further studies using metagenomic approaches could provide deeper insights into microbial succession and metabolic activities during fermentation.

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