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Effect of processing methods on cyanogenic compounds, protein, and minerals of cassava leaves

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Abstract

This study investigated the effects of boiling, drying, and ensiling on linamarin content, protein content, and mineral composition of cassava (*Manihot esculenta* Crantz) leaves. The drying was found effective method for reducing linamarin content in most genotypes. Boiling had variable effects, with some genotypes showing similar or slightly lower linamarin levels compared to fresh leaves. Ensiling significantly reduced linamarin content after 7 days, with the reduction remaining stable up to 20 days, indicating its effectiveness in reducing cyanogenic compounds. However, genotypes, H 1687 and Malayan 4, showed a decrease in protein content during ensilage, suggesting potential nutritional changes. The study also observed dynamic changes in mineral composition of cassava leaves, with copper, zinc, iron, and manganese varying over time. These findings highlight the impact of different processing methods on nutritional quality of cassava leaves, emphasizing the need to consider these factors when using cassava leaves as animal feed.

Key words: Cyanogenic compounds, Genotype, Linamarin, Processing methods, Leaves

Cassava (*Manihot esculenta* Crantz) is a crucial staple food crop, extensively cultivated for its starchy roots (Suchitra and Byju, 2015; Soman and Byju, 2017). However, increasing attention has been given to the nutritional value of cassava leaves. Despite their nutritional benefits, cassava leaves contain anti-nutritional cyanogens (Nedunchezhiyan *et al.,* 2022; Gundersen *et al.,* 2022). Studying the distribution and levels of linamarin, a primary cyanogenic glycoside in various cassava genotypes is essential for evaluating the potential health risks of consuming cassava products (Ndam *et al.,* 2019). This knowledge supports the development of breeding strategies and the selection of cassava genotypes with reduced linamarin content (Sayre, 2022). Urgent interventions and regulations are needed to raise awareness about the health risks associated with cyanogens in cassava leaves and to promote proper processing methods to mitigate these risks (Okareh *et al.,* 2021). Microbial enzymes play a crucial role in converting cyanogens into less toxic compounds, resulting in significantly lower cyanide levels (Mahendran *et al.,* 2020). This study investigates the potential of fermentation, boiling, and drying in reducing cyanogen levels in cassava leaves, aiming to enhance cassava-based foods' safety and nutritional value.

Materials and Methods

Ten genotypes of cassava leaves were chosen from the field of ICAR-CTCRI, Thiruvananthapuram, Kerala during 2018-19. They were H-226, Sree Athulya, Me-833, Quintal, M-4 (Malayan), Sree Jaya, Sree Vijaya, H-1687 (Sree Visakam), Sree Swarna, and Ci-848. First, a representative sample of cassava leaves was collected. The sample was then weighed using a digital balance, and the weight was recorded as the fresh weight. The fresh sample was then placed in an oven set at a specific temperature (70°C) and left to dry, and the weight was recorded as the dry weight.

For the preparation of cassava leaf silage, 5 kg of fresh cassava leaves from Malayan 4 and H-1687 varieties were collected. The leaves were allowed to wilt for 12 hours until they reached a moisture content of 70%. Subsequently, wilted leaves were chopped into 10 mm pieces using a hand-operated chopping machine. The chopped leaves were weighed and mixed thoroughly with 50 g of activated encapsulated yeast (*Saccharomyces cerevisiae*) in a concentrated sugar

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solution, along with the addition of 10 g of urea. The inclusion of sugar helps accelerate the fermentation process. The mixture was well-blended and packed tightly into polypropylene plastic bags measuring 60 cm in width and 100 cm in length, ensuring the expulsion of air from the bags. Rubber bands were used to secure the bags, which were then placed in anaerobic conditions for 3-4 weeks. The mineral and nutrient content of the silage was monitored during the first, second, and third weeks, and the quality of the silage was compared to that of the fresh leaves.

Linamarase, enzyme responsible for catalyzing the breakdown of linamarin in cassava, was isolated from cassava latex obtained from the cut end of cassava petioles. The isolation process involved acetone and ammonium sulfate precipitation. Two g of cassava latex were mixed with 50 ml of phosphate buffer (pH 6.0) and stirred for 30 minutes. The solution was then centrifuged at 10,000 × g for 30 minutes, and the clear supernatant was divided into two equal fractions. In one fraction, chilled acetone was added in three times the volume, and the solution was refrigerated overnight. The precipitated proteins were collected by centrifugation, dissolved in 10 ml of phosphate buffer (0.1 M, pH 6.0), and stored at 4°C for further use. The second fraction, after removing cell debris, was subjected to 60% saturation ammonium sulfate and kept at 4°C overnight. The resulting precipitate was dissolved in 10 ml of phosphate buffer, extensively dialyzed against diluted buffer, and used for enzyme activity assay.

Samples of fresh, boiled, dried, and ensiled leaves were obtained and weighed (2 g). The samples were then homogenized with 25 ml of orthophosphoric acid using a mortar and pestle. After centrifugation at 5000 rpm for 15 minutes, the supernatant was collected. The residue was subjected to an additional extraction in 25 ml of orthophosphoric acid, and the resulting supernatants were combined and adjusted to a final volume of 50 ml. In test tubes, 0.1 ml of the supernatant was mixed with 0.4 ml of phosphate buffer (0.1 M, pH 7.0) and 0.5 ml of phosphate buffer (pH 6.0), making a total volume of 1 ml. Then, 20µl of the linamarase enzyme was added to tubes, which were incubated at 30˚C for 15 minutes. The reaction was halted by adding 0.6 ml of 0.2N NaOH and thorough mixing. After 1 minute, 2.8 ml of phosphate buffer (pH 6.0) was added and shaken. To this mixture, 0.1 ml of chloramine T solution was added, followed by the addition of 0.6 ml of iso-nicotinic acid barbiturate coloring reagent. The resulting pink complex was incubated for 10 minutes and its absorbance was measured at 605 nm.

The protein estimation was performed using the Lowry method. A standard protein solution was prepared from bovine serum albumin (BSA) at 1 mg/ml concentration. Dilutions were made ranging from 0.05 to 1 mg/ml. Each protein solution (0.2 ml) was placed in separate tubes. An alkaline copper sulfate reagent was formed by combining 2% sodium carbonate and 0.1 N NaOH (solution A), along with 1.56% copper sulfate and 2.37% sodium potassium tartrate (solution B). These solutions were mixed to create the reagent. Tubes received 2 ml of the reagent and were incubated for 10 minutes. Following this, 0.2 ml of Folin-Ciocalteu solution (diluted from commercial reagent) was added to each tube and incubated for an additional 30 minutes. The resulting purple color's absorbance was measured at 660 nm using a UV-visible spectrophotometer. A calibration curve was constructed using absorbance values and known protein concentrations. The absorbance of an unknown sample, treated similarly, was measured at 660 nm to determine its protein concentration using the calibration curve. The protein content value is expressed on a dry weight basis.

For the analysis of mineral content, dried powdered leaf samples were used. In test tubes, 0.5 g of the samples were combined with 15 ml of a tri-acid mixture consisting of nitric acid (HNO_3), perchloric acid ($HClO_4$), and sulphuric acid (H_2SO_4) in a ratio of 10:4:1. The tubes were left overnight in a beaker containing a small amount of sulphuric acid for wet digestion. After wet digestion, the samples underwent hot digestion on a hot plate at 125° C for approximately 2 hours to ensure complete digestion. Following cooling, the clear sample digest was taken and diluted to a final volume of 100 ml using distilled water. The tri-acid digest, obtained from the sample, was utilized for the estimation of Ca, Mg, Fe, Mn, Zn, and Cu using an Atomic Absorption Spectrophotometer (AAS).

The phosphorous content in leaf samples was determined using a calorimetric method with a spectrophotometer (Double beam spectrophotometer 2202, Systronics). A volume of 5 ml of the tri-acid digest was transferred to a 25 ml standard flask. To this, 5 ml of Vanadomolybdate reagent was added, and the final volume was adjusted to 25 ml with distilled water. The resulting sample solution was allowed to sit for one hour to develop color. The yellow color that formed was then measured at a wavelength of 420 nm

using the spectrophotometer, and absorbance value was recorded. The potassium (K) content in leaf samples was determined using a flame photometer. For analysis, 1 ml of tri-acid digest was transferred to a standard flask and diluted to a final volume of 25 ml. The resulting extract was directly measured using a flame photometer (Systronics Flame Photometer Model No. 128), utilizing the appropriate filter for potassium.

To assess the quality of cassava leaf silage, samples from H 1687 and Malayan 4 varieties enriched on day 7 and day 20 were selected. The protein, linamarin, and mineral contents were the parameters measured in this study. The procedures mentioned earlier were followed to determine these parameters. The samples were dried in an oven and then transformed into a powdered form for analysis.

Results and Discussion

The variation in linamarin content was investigated in fresh, dry, and boiled cassava leaf samples (Fig.1). The linamarin content was analyzed in the fresh leaf tissue of various cassava genotypes and among the samples, Sree Jaya exhibited the lowest linamarin content, measuring at 0.75 ± 0.20 mg/g (Fig.1). Slightly higher linamarin levels were observed in H 226, Malayan 4 and Sree Vijaya, with values of 1.24 ± 0.1 mg/g, 1.32 ± 0.36 mg/g and, 1.72 ± 0.44 mg/g, respectively. The genotype Quintal displayed the highest linamarin content, recording a value of 6.04 ± 1.69 mg/g. Sree Athulya demonstrated the second-highest linamarin content among all genotypes tested, measuring at 4.40 ± 0.05 mg/g. Overall, the observations in fresh leaves demonstrated significant variance in linamarin content among the investigated cassava genotypes.

Boiling cassava generally resulted in a decrease in linamarin content (Fig.1), as observed in most genotypes compared to fresh leaves. Sree Jaya, Sree Vijaya, Malayan 4, H 226, Sree Athulya, Sree Swarna, and Ci 848 showed lower linamarin levels in boiled samples compared to the fresh ones. H 1687 and Me 833 exhibited a moderate decrease in linamarin content after boiling. Quintal and Me 833 displayed the highest linamarin levels among the genotypes, with values of 1.20 ± 0.06 mg/g and 1.077 ± 0.39 mg/g, respectively. On the other hand, Ci 848 and Sree Jaya exhibited the lowest linamarin content at 0.34 ± 0.01 mg/g and 0.39 ± 0.01 mg/g.

Drying had variable effects, with some genotypes showing similar or slightly lower linamarin levels in dried samples compared to the boiled ones (Fig.1). Among the genotypes tested, Sree Jaya, Sree Vijaya, Malayan 4, H 226, Sree Athulya, Quintal, Sree Swarna, and Ci 848 exhibited similar linamarin levels in the dried samples, ranging from 0.34 ± 0.02 mg/g to 0.41 ± 0.01 mg/g. The H 1687 and Me 833 genotypes showed a slightly higher linamarin content compared to the aforementioned genotypes, with a value of 0.51 ± 0.06 mg/g and 0.44 ± 0.06 mg/g, respectively. The linamarin

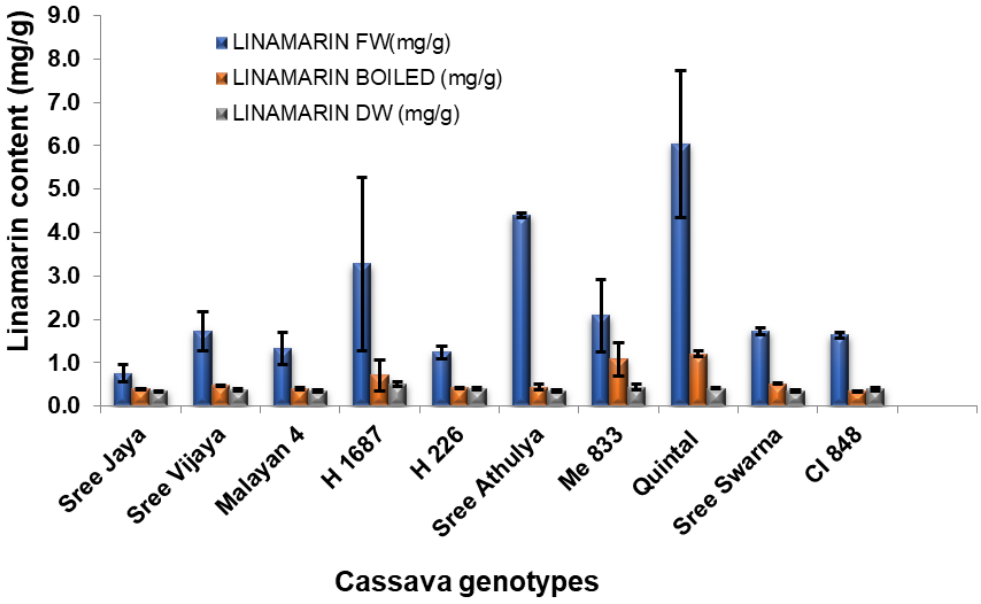

Fig. 1: Variation in linamarin content in fresh (FW), dried (DW), and boiled leaf samples of cassava genotypes

content was found lowest in Sree Swarna, Malayan 4 and Sree Athulya genotypes with the values 0.34 ± 0.02 mg/g, 0.35 ± 0.01 mg/g and 0.35 ± 0.02 mg/g, respectively. The analysis of linamarin content in various cassava genotypes revealed significant variation. These findings are consistent with previous studies that have highlighted the variability in cyanogenic potential among different cassava genotypes (Chaiareekitwat *et al.,* 2022) and useful for selection of best genotype for indeed applications (Swaroop *et al.,* 2019). Overall, drying was more effective in reducing linamarin content in cassava compared to boiling and fresh leaves samples. The drying and boiling effects on linamarin content in cassava have been widely studied. The drying cassava roots resulted in less linamarin breakdown compared to boiling (Ndubuisi and Chidiebere, 2018). However, other studies have shown that drying with higher moisture levels can enhance linamarin breakdown (Bolarinwa *et al.,* 2016). Drying at elevated temperatures has been found to promote linamarin degradation, leading to a decrease in linamarin content (Junior *et al.,* 2019). On the other hand, boiling cassava generally leads to a reduction in linamarin content, as observed in most genotypes (Panghal *et al.,* 2019). The high temperature during boiling treatment results in the inactivation of heat-sensitive enzymes such as linamarase, which is responsible for the degradation of linamarin (Panghal *et al.,* 2019). The boiling cassava roots at temperatures above the boiling point of HCN facilitates the release of HCN, contributing to linamarin degradation (Ndubuisi and Chidiebere, 2018). The formation of HCN during boiling might be attributed to the thermal degradation of cyanohydrins, which are intermediates in the breakdown of linamarin.

The linamarin contents of ensilaged leaf samples for genotypes H1687 and Malayan 4 were studied and analyzed on the 7th and 20th day of ensilage. The initial linamarin content (mg/g) of H1687 was 3.27 ± 1.99, while for Malayan 4, it was 1.32 ± 0.36 on day 0 (Table 1). However, after 7 days of ensilage, the linamarin content significantly decreased for both genotypes, with H1687 showing a content (mg/g) of 0.35 ± 0.001 and Malayan 4 exhibiting 0.36 ± 0.03. By the 20th day of ensilage, the linamarin content (mg/g) remained relatively stable, with H1687 measuring at 0.35 ± 0.01 and Malayan 4 at 0.36 ± 0.001. There was a decrease in linamarin content during the ensilage period for both genotypes, suggesting a potential reduction in cyanogenic compounds in the ensilaged leaf samples. These findings align with previous studies that have demonstrated the effectiveness of ensiling in reducing cyanogenic compounds in cassava biomass (Unigwe *et al.,* 2023). The observed decrease in linamarin content during ensilage highlights the hydrolysis process, wherein linamarin is converted into less toxic compounds. This outcome has important implications for the safety and utilization of cassava leaf biomass in monogastric animal diets. Thus, ensilage can be considered a viable method for reducing linamarin content and enhancing the safety of cassava leaf biomass for animal feed applications.

In addition, the genotypes were tested to find out more about the influence of ensilaged leaf samples on cassava nutrition. Both the H 1687 and Malayan 4 (M4) genotypes of cassava leaf ensilage experienced a reduction in protein content on both the 7th and 20th days (Table 1). Specifically, the protein content of H 1687 decreased from an initial value of 0.28 ± 0.02 g/g to 0.24 ± 0.018 g/g on the 7th day and further to 0.19 ± 0.012 g/g on the 20th day. Similarly, the protein content of Malayan 4 decreased from the initial value of 0.28 ± 0.019 g/g to 0.22 ± 0.017 g/g on the 7th day and then to 0.20 ± 0.01 g/g on the 20th day.

The mineral analysis of cassava leaf genotypes, Malayan 4 and H 1687, on the 0th, 7th, and 20th day of enrichment revealed dynamic changes in copper (Cu), zinc (Zn), iron (Fe), and manganese (Mn) content (Fig.2). The copper content in Malayan 4 increased from 28.3 mg/l initially to 34.3 mg/l (7th day) and 44.6

Table. 1: Changes in linamarin and protein content of cassava leaf silage

Genotype	Days after treatment	Linamarin content (mg/g)	Protein content (g/g)
H1687	0	3.27 ± 1.99	0.28 ± 0.02
	7	0.35 ± 0.001	0.24 ± 0.018
	21	0.35 ± 0.01	0.19 ± 0.012
Malayan 4	0	1.32 ± 0.36	0.28 ± 0.019
	7	0.36 ± 0.03	0.22 ± 0.017
	21	0.36 ± 0.001	0.20 ± 0.01

mg/l (20th day), while H 1687 showed an increase from 28.5 mg/l to 46.2 mg/l (7th day) and then a decrease to 33.2 mg/l (20th day). Zinc content decreased over time for both genotypes, with Malayan 4 dropping from 176.2 mg/l to 167.2 mg/l (7th day) and 127 mg/l (20th day), and H 1687 decreasing from 230.9 mg/l to 166.1 mg/l (7th day) and 144.3 mg/l (20th day). Iron content was found 476.7 mg/l and 475.4 mg/l for Malayan 4 and H 1687, respectively on 0th day, the content decreased on the 7th day, reaching 397.1 mg/l (Malayan 4) and 379.7 mg/l (H 1687), followed by a slight increase to 433.4 mg/l (Malayan 4) and 394 mg/l (H 1687) on the 20th day. Manganese content decreased to 117.2 mg/l (Malayan 4) and 162.8 mg/l (H 1687) on the 7th day, and then increased to 160 mg/l (Malayan 4) and decreased to 104.2 mg/l (H 1687) on the 20th day. Initially the manganese content was observed as 311.5 mg/l and 222.7 mg/l for Malayan 4 and H 1687, respectively on 0th day. These results highlight the dynamic nature of mineral content during the enrichment process for these cassava genotypes. The phosphorus (P) content in the leaf samples was relatively low, with Malayan 4 and H 1687 exhibiting initial values of 2.467 mg/l and 2.577 mg/l, respectively. On the 7th day of enrichment, the phosphorus content increased to 2.913 mg/l (Malayan 4) and 2.241 mg/l (H 1687), and further 2.86 mg/l (Malayan 4) and 2.66 mg/l (H 1687) on the 20th day.

Changes in mineral content of potassium (K), calcium (Ca), and magnesium (Mg) in cassava leaf silage were also investigated (Fig.3). Regarding potassium content, Malayan 4 started with 14150 mg/l and decreased to 9075 mg/l (7th day) and then increased to 10600 mg/l (20th day), while H 1687 started with 19275 mg/l and decreased to 12025 mg/l (7th day) and 7650 mg/l (20th day). Calcium content showed a decrease for both genotypes, with Malayan 4 dropping from 9127.5 mg/l to 5765 mg/l (7th day) and then increased to 6630 mg/l (20th day), and H 1687 dropping from 8462.5 mg/l to 7532.5 mg/l (7th day) and 6465 mg/l (20th day). Similarly, magnesium content decreased for both genotypes, with Malayan 4 dropping from 6977.5 mg/l to 3952.5 mg/l (7th day) and increasing to 5290 mg/l (20th day), and H 1687 dropping from 6037.5 mg/l to 5135 mg/l (7th day) and 4472.5 mg/l (20th day).

Based on the provided results, the mineral content of cassava leaves, specifically copper, zinc, iron, manganese, phosphorus, potassium, calcium, and magnesium, exhibited dynamic changes during the enrichment process. These findings are consistent with previous studies on the mineral composition of cassava leaves, which have reported variations in mineral content at different growth stages and during maturity (Laya *et al.,* 2023). The observed changes in mineral content could be attributed to factors such as plant

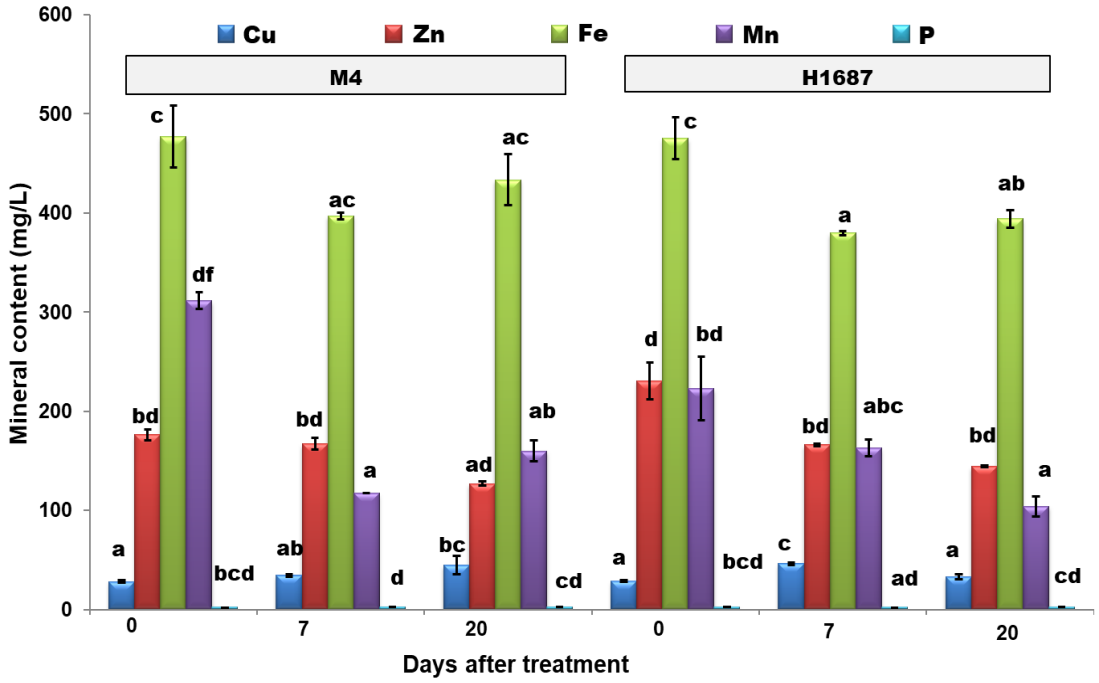

Fig. 2: Changes in mineral content (Cu, Zn, Fe, Mn and P) in cassava leaf silage

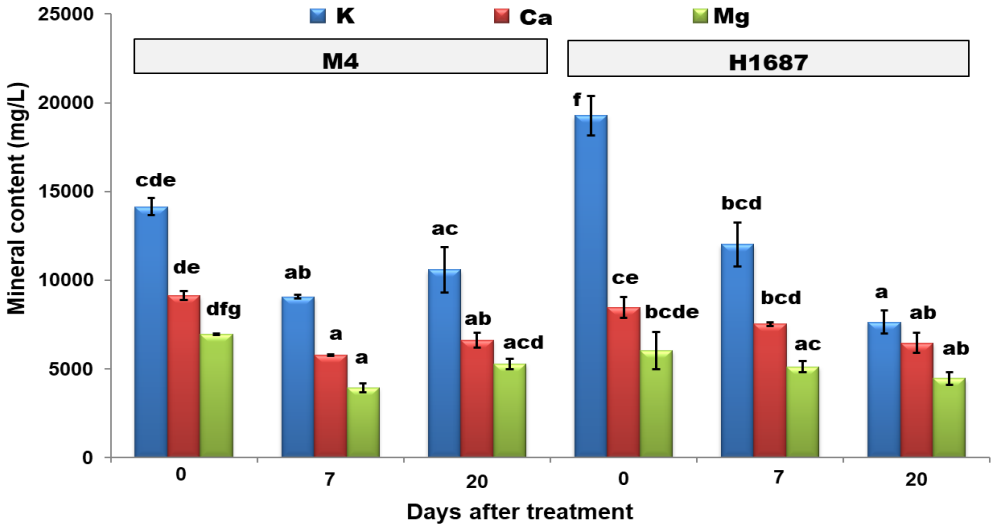

Fig. 3: Changes in mineral content (K, Ca, Mg) in cassava leaf silage

maturity, nutrient availability, and the physiological processes occurring during leaf development. Additionally, the use of ensilage techniques and additives, as highlighted in studies involving cassava (Oduguwa *et al.,* 2007), may have influenced the mineral content changes during the enrichment process. These results emphasize the importance of considering the mineral composition of cassava leaves and their variations over time when evaluating their nutritional value and potential utilization as animal feed (da Silva Santos *et al.,* 2020).

Conclusion

This study reveals that ensilage and drying cassava effectively reduces linamarin levels while boiling also contribute to the reduction of cyanogenic compounds. Protein content decreased during ensilage, indicating potential changes in nutritional value. The mineral composition of cassava leaves exhibited dynamic changes during the enrichment process, emphasizing the need to consider these variations when assessing their nutritional value. These findings provide valuable insights into the safe utilization of cassava leaves as animal feed, highlighting the importance of appropriate processing methods to enhance their safety and nutritional quality. Further research is warranted to explore additional processing techniques and their effects on cassava leaf composition.

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