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Rapidly fermentable carbon accelerates biofermentation, reduces anti-nutrients, and improves the nutritional value of *Chromolaena odorata*

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ABSTRACT. The aim of the experiment was to evaluate the effect of biofermentation duration of fresh *Chromolaena odorata* leaves, using a readily soluble carbon source, on nutrient content, digestibility, and concentrations of anti-nutritional compounds. A completely randomised design (CRD) was used to assess the effect of four treatments. *Chromolaena odorata* leaves were supplemented with a soluble carbon source (liquid sugar), and either not fermented (ICS0), or fermented for 7 days (ICS7), 14 days (ICS14), and 21 days (ICS21). The experimental variables included dry matter (DM), organic matter (OM), crude protein (CP), crude lipid (CL), lignin, haemicellulose, cellulose, acid detergent fibre (ADF), neutral detergent fibre (NDF), phytic acid, oxalate, saponin, and nitrite. Additional variables were *in vitro* digestibility of DM, OM, CP, and CL. Results showed that biofermentation time significantly increased DM, OM, CL, total carbohydrate, metabolizable energy, and *in vitro* digestibility of DM and OM. Conversely, a highly significant reduction ($P < 0.01$) was observed in all fibre fractions and anti-nutritional compounds. Improvements in nutrient composition and digestibility, together with reductions in fibre fractions and anti-nutritional compounds, were most pronounced after 7 days of incubation, followed by slowdown in the rate of change. Calcium and phosphorus concentrations, as well as the content and digestibility of CP and CL, were unaffected by incubation time. It was concluded that the optimal bio-fermentation period for *C. odorata* using liquid sugar as a soluble carbon source was 7 days, as this treatment resulted in the greatest improvement in nutritional value and the largest reduction in anti-nutritional compounds.

Introduction

Chromolaena odorata, locally known as white flower bush, is regarded as one of the most invasive weed species worldwide, causing severe degradation of native pastures in tropical regions. Nevertheless, this plant possesses considerable potential as an alternative feed resource due to its relatively high dry matter (DM) yield, reaching approximately 70 t/ha/year, with crude protein (CP) content ranging from 21 to 36% and metabolizable energy (ME) of approx. 2 909 kcal/kg DM (Mullik et al., 2024). In addition, its amino acid profile is characterised by a relatively favourable composition and balance (Fasuyi et al., 2005; Oematan, 2020). However, *C. odorata* limitations include a relatively high lignin (13.1%) and cellulose (40.2%) contents, a low methionine concentration (0.01%)

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(Oematan et al., 2020), and the presence of various secondary metabolites that act as anti-nutritional factors. These include nitrate, nitrite, alkaloids, cyanogenic glycosides, flavonoids (aurones, chalcones, flavones, and flavonols), phytic acid, saponins, tannins, and trypsin inhibitors (Ngozi et al., 2009; Akinmoladun et al., 2010; Kato-Noguchi and Kato, 2023). Such compounds reduce palatability, total intake, *in vivo* digestibility, and cattle growth performance (Mullik et al., 2015; Bira et al., 2017; Oematan, 2020; Oematan, 2023; Oematan et al., 2024).

Biofermentation is widely used to overcome the limitations of *C. odorata* as a feed resource, including through silage production (Ridla et al., 2016; Mulik et al., 2016; Oematan et al., 2023) and fermentation based on carbon-to-nitrogen (C:N) ratio principles utilising carbon sources with fast, intermediate, or slow fermentation rates (Mullik et al., 2019; Oematan, 2020). Both silage production, using rice bran as an additional energy source, and fermentation based on the C:N ratio with different carbon sources, successfully improved nutritional quality, digestibility, and reduced secondary metabolite contents in *C. odorata*.

However, a major drawback of these approaches is the relatively long fermentation period, which requires at least 21 days and thus reduces practical efficiency. Furthermore, studies employing different carbon sources with varying fermentation rates (Oematan, 2020) showed that liquid palm sugar, which was expected to provide a rapidly available carbon source, had a smaller effect compared to rice bran and rice straw flour. It was hypothesised that the carbon supplied by liquid sugar was depleted too rapidly during the initial fermentation phase, leaving insufficient energy to support microbial activity throughout the biofermentation.

Accordingly, the present study evaluated the effect of liquid palm sugar as a rapidly soluble carbon source in the biofermentation of *C. odorata* for periods shorter than 21 days, focusing on its impact on nutrient composition and *in vitro* digestibility, rumen fermentation products, and the concentration of secondary metabolites in the fermented material.

Material and methods

All procedures applied in this study were approved by the Livestock, Marine and Fishery Research Ethics Committee of Nusa Cendana University (Ref. No. 0143/1.KT/KEPPKP/VII/2024).

The present *in vitro* study employed a completely randomised design to evaluate the effects of four biofermentation periods of *C. odorata*: 21 days (ICS21, control), 14 days (ICS14), 7 days (ICS7), and no incubation (ICS0). ICS refers to incubated (I) *Chromolaena* (C) with sugar (S). Each treatment was carried out in five replicates. All treatments were supplemented with a specified amount of liquid palm sugar as a source of rapidly fermentable carbohydrate to obtain a C:N ratio of 30. In addition, 1% fresh rumen fluid was added as a starter inoculum to accelerate biofermentation. The C:N ratio was calculated according to the method described by Jimenez and Garcia (1997). The 21-day incubation period was selected as the control because it was the longest period evaluated by Oematan (2020).

Experimental procedures

Fresh whole *C. odorata* plants were, collected from an open area near the experimental site and hand-chopped into 2–3 cm fragments. The collected material was spread evenly on a plastic-covered floor to a thickness of 5–6 cm and air-dried for 24 h before treatment. After drying, 100 kg of the material was weighed and thoroughly mixed with liquid palm sugar (a rapidly fermentable carbohydrate) and fresh rumen fluid at rates of 10 ml and 47 ml/kg DM of *C. odorata*, respectively. The mixture was subsequently packed into 20 plastic containers, each with a capacity of 5 kg, which served as silos. The containers were tightly closed and sealed to ensure anaerobic conditions. In the next step, the silos were randomly assigned to the four treatments: fermentation for 21 days (ICS_{21}, control), 14 days (ICS_{14}), 7 days (ICS_{7}), and 0 days (ICS_{0}).

Variables measured included the content and *in vitro* digestibility of DM, organic matter (OM), CP, ether extract (EE), crude fibre (CF), cholesterol (CHO), Ca, P, neutral detergent fibre (NDF), acd detergent fibre (ADF), lignin, cellulose, gross energy (GE), and ME, as well as the concentrations of saponin, phytic acid, oxalate, and nitrite.

Nutrient concentrations were determined using proximate analyses described in AOAC (2005) procedures. NDF, ADF, lignin, and cellulose were quantified according to the methods of Van Soest (1985). GE and ME were estimated using the equations of Hvelplund et al. (1995).

In vitro digestibility was determined the method of Tilley and Terry (1963). Sampl using es for *in vitro* digestion were collected from all experimental units after biofermentation. Digesta pH was measured at the end of the digestion process using a Hanna digital pH meter (HI83141-1; Hanna Instruments, India). N-ammonia concentra-

tion was determined according to the procedure of Millar (1966).

Phytic acid concentration was determined by a colorimetric method using Wade's reagent ($FeCl_3$ – sulphosalicylic acid complex), with absorbance measured at 500 nm (Iwuozor, 2019). Oxalate concentration was quantified using a UV-Vis spectrophotometric method involving acid extraction, precipitation of oxalate as calcium oxalate, and conversion to oxalic acid, followed by absorbance measurements at 627 nm after reaction with potassium permanganate (Salgado et al., 2023). Total saponins were determined using the vanillin–sulphuric acid assay (Le et al., 2018). Nitrite concentration was determined according to the AOAC International (2005) standard analytical procedure using spectrophotometry. A Cary 50 UV-Vis spectrophotometer (Agilent Technologies, Santa Clara, CA, USA) was used for all spectrophotometric measurements.

Data analysis

Data were analysed using analysis of variance for a completely randomised design. Treatment effects on the measured variables were assessed at the 5% significance level. Differences among treatments were further evaluated using Duncan's multiple range test. All statistical analyses were performed using IBM SPSS Statistics for Windows, version 27 (IBM, 2020).

Results

Nutrient content

The nutrient composition of biofermented *C. odorata* presented in Table 1 demonstrates that the use of liquid palm sugar as a readily available carbon source during biofermentation, combined with different fermentation periods, significantly affected ($P < 0.05$) DM, OM, EE, total CHO, and ME. The lowest DM content (87.58%) was observed after 7 days of incubation (ICS_7), whereas the highest value (91.63%) was recorded after 14 days (ICS_{14}). For OM, the highest content (90.71%) occurred in ICS_7, while the lowest (88.41%) was found in ICS_0. EE ranged from 7.30 to 9.64%, with the highest value recorded in ICS_{21}. Total CHO increased significantly from 60.21 in ICS_0 to 62.85% in ICS_7. The elevated levels of total CHO and EE in ICS_7 and ICS_{21} were associated with correspondingly high ME values of 3 380 cal/kg DM and 3 447 cal/kg DM, respectively. CP (19.57–20.78%), Ca (0.82–0.92%), and P (0.51–0.54%) were not affected by fermentation period ($P > 0.05$).

Fibre and its fractions

Total CF and fibre fractions of *C. odorata* biofermented with liquid palm sugar as carbon source for different incubation periods are presented in Table 2. Total CF ranged from 17.37 (ICS_7) to 22.1% (ICS_0). These relatively high CF values were expected because the whole plant, consisting of both stems and leaves, was used as the fermentation substrate. Therefore, a reduction in CF content was likely to improve feed degradability. Incubation period had a highly significant effect on total CF. However, a marked reduction occurred by day 14 of incubation (Table 2), whereas extending the incubation period to 21 days (ICS_{21}) produced no further reduction in total CF.

Nutrient digestibility

Table 3 shows that *in vitro* crude protein digestibility *in vitro* (*i*CPD) and *in vitro* crude lipid digestibility (*i*CLD) were not affected ($P > 0.05$) by the duration of *Chromolaena* incubation, as opposed to *in vitro* organic matter digestibility (*i*OMD) and

Table 1. Nutrient content of biofermented *Chromolaena odorata* using a fermentable carbohydrate as source of carbon, %

Variable	Treatment				SEM	*P*-value
	ICS_{21}	ICS_{14}	ICS_7	ICS_0		
Dry matter (DM)	9.419[b]	90.63[b]	87.58[a]	89.96[b]	0.438	0.018
Organic matter	90.48[b]	89.61[b]	90.71[b]	88.41[a]	0.150	0.019
Crude protein	19.57	19.92	19.96	20.78	1.012	0.682
Ether extract	7.64[b]	7.50[b]	7.83[b]	7.13[a]	0.174	0.041
Total carbohydrates	61.26[b]	62.49[b]	62.85[b]	60.21[a]	0.798	0.023
Metabolizable energy, cal/kg DM	3 447[b]	3 125[a]	3 380[b]	3 070[a]	54.85	0.012
Calcium	0.82	0.86	0.92	0.92	0.040	0.105
Phosphorus	0.51	0.51	0.54	0.54	0.029	0.486
pH	6.36	6.46	6.38	6.48	0.078	0.170
$N\text{-}NH_3$, mM	10.77[a]	9.61[a]	10.89[a]	12.54[b]	0.68	0.012

CS_{21} – fermented for 21 days, ICS_{14} – fermented for 14 days; ICS_7 – fermented for 7 days; ICS_0 – not fermented; SEM – standard error of the means; [ab] – values within a row with different superscripts are significantly different at $P < 0.05$

Table 2. Fibre content of biofermented *Chromolaena odorata* using a rapidly fermentable carbohydrate as source of carbon, %

Variable	Treatment				SEM	*P*-value
	ICS_{21}	ICS_{14}	ICS_{7}	ICS_{0}		
Total crude fibre	17.54[a]	17.37[a]	22.04[b]	22.1[b]	0.767	0.001
Fibre fractions:						
lignin	9.99[a]	10.53[a]	12.23[b]	13.80[c]	0.037	0.001
cellulose	20.51[a]	21.12[a]	20.23[a]	23.11[b]	0.786	0.001
haemicellulose	10.49[a]	16.84[b]	17.15[b]	22.34[c]	2.295	0.001
neutral detergent fibre	39.89[a]	48.49[b]	49.61[b]	59.25[c]	2.090	0.001
acid detergent fibre	29.4[a]	31.65[a]	32.46[a]	36.91[b]	1.996	0.001

CS_{21} – fermented for 21 days, ICS_{14} – fermented for 14 days; ICS_{7} – fermented for 7 days; ICS_{0} – not fermented; SEM – standard error of the means; [ab] – values within a row with different superscripts are significantly different at $P < 0.05$

Table 3. *In vitro* nutrient digestibilities of biofermented *Chromolaena odorata* using a rapidly fermentable carbohydrate as source of carbon, %

Variable	Treatment				SEM	*P*-value
	ICS_{21}	ICS_{14}	ICS_{7}	ICS_{0}		
Dry matter digestibility	70.20[b]	63.35[a]	67.35[b]	61.10[a]	2.084	0.012
Organic matter digestibility	71.61[b]	64.07[a]	68.45[b]	62.05[a]	1.941	0.013
Crude protein digestibility	77.58	77.87	77.83	78.54	0.771	0.653
Crude lipid digestibility	84.68	82.44	82.82	81.92	1.709	0.435

CS_{21} – fermented for 21 days, ICS_{14} – fermented for 14 days; ICS_{7} – fermented for 7 days; ICS_{0} – not fermented; SEM – standard error of the means; [ab] – values within a row with different superscripts are significantly different at $P < 0.05$

dry *in vitro* matter digestibility *i*DMD, which were significantly influenced by biofermentation time ($P < 0.05$).

Significant differences were observed between treatments ($P = 0.013$). ICS_{21} (71.61%) and ICS_{7} (68.45%) showed significantly higher values compared to ICS_{14} (64.07%) and ICS_{0} (62.05%). This suggested that biofermentation improved OM digestibility, particularly after 14 and 21 days of incubation. Similarly, iDMD differed significantly among treatments ($P = 0.012$). ICS_{21} (70.20%) and ICS_{7} (67.35%) showed significantly higher digestibility than ICS_{14} (63.35%) and ICS_{0} (61.10%).

In contrast to *i*BMD and *i*OMD, *i*CP and crude lipid (*i*CLD) were not affected by biofermentation time. The *i*CPD values ranged from 77.58 (ICS_{0}) to 78.54% rapidly (ICS_{21}), whereas *i*CLD were from 81.9 (ICS_{0}) to 84.68% (ICS_{21}).

Anti-nutritional properties

The concentrations of all four anti-nutritional compounds (saponin, phytate, oxalate, nitrite) decreased significantly with increasing incubation periods (Table 4). The most pronounced reductions occurred between 14 and 21 days, indicating that extended incubation was effective in reducing anti-nutritional factors. Saponin decreased steadily from 7.70 in ICS_{0} to 5.80% in ICS_{21}. Phytate declined sharply from 3.91 (ICS_{0}) to 1.88% (ICS_{21}). Oxalate declined from 0.54 (ICS_{0}) to 0.42% (ICS_{21}), whereas nitrite from 6.59 (ICS_{0}) to 4.70 ppm (ICS_{21}). Incubation period had highly significant effects on all variables ($P = 0.001$). These results suggest that incubation can improve the nutritional

Table 4. Anti-nutritional compounds in *Chromolaena odorata* using a rapidly fermentable carbohydrate as source of carbon, %

Variable	Treatment				SEM	*P*-value
	ICS_{21}	ICS_{14}	ICS_{7}	ICS_{0}		
Saponin	5.80[a]	6.08[b]	6.47[c]	7.70[d]	0.039	0.001
Phytate	1.88[a]	2.26[a]	3.59[b]	3.91[b]	0.222	0.001
Oxalate	0.42[a]	0.45[a]	0.49[b]	0.54[c]	0.016	0.001
Nitrite, ppm	4.70[a]	4.92[a]	5.80[b]	6.59[c]	0.120	0.001

CS_{21} – fermented for 21 days, ICS_{14} – fermented for 14 days; ICS_{7} – fermented for 7 days; ICS_{0} – not fermented; SEM – standard error of the means; [ab] – values within a row with different superscripts are significantly different at $P < 0.05$

quality of *C. odorata* by lowering the levels of compounds that limit intake, digestibility or nutrient absorption.

However, 14 days of incubation (ICS_{14}) appeared to be the most effective treatment, as extending the incubation period to 21 days (ICS_{21}) did not result in further significant reductions compared to ICS_{14}.

Discussion

Nutrient and fibre content

The low DM content (87.58%), accompanied by the high OM (90.71%), EE (7.83%), total CHO (62.85%), and ME (3,380 kcal/kg DM) values observed in ICS_7 was likely attributable to intensive degradation of the substrate (*C. odorata*) during biofermentation. Two factors may have contributed to this response. Firstly, liquid sugar represents a readily fermentable non-structural carbohydrate (Hoover et al., 2006; Li et al., 2025a), which rapidly supplies carbon for microbial growth, thereby stimulating microbial proliferation and increasing the rate of substrate degradation (Mullik et al*.,* 2024). Indeed, faster degradation of non-structural carbohydrates has been shown to increase nutrient digestion (Lykos et al*.,* 1997). Secondly, synchronisation may occur between the release of carbon (energy) from liquid sugar and the release of nitrogen (protein) from the substrate, as protein fractions of *C. odorata* are highly soluble. Using the *in sacco* technique, Mullik et al. (2024) demonstrated that 54–60% of protein in *C. odorata* disappeared within 72 h of incubation.

Intensive degradation of complex substrates by fermentative microorganisms during the initial 7 days of incubation (ICS_7) releases water bound within the substrate, thereby increasing the proportion of free water molecules. Wang et al. (2024) reported that water content rises in the early stage of biofermentation due to metabolic water production and enzymatic hydrolysis. Conversely, extending the fermentation period tends to reduce water content. Daia et al. (2020) and Siddik et al. (2024) observed that water released from the substrate during the initial stage was subsequently utilised by fermentative microbial populations for their cell synthesis and growth. Considering the short lifespan of anaerobic fermentative microorganisms, which ranges from several hours to a few days, depending on the microbial species and the availability of protein and energy (Yin et al., 2021), microbial turnover during a 21-day biofermentation period was likely high. Consequently, water utilisation may have increased in parallel with microbial population alterations, leading to the lower free water content observed in the ICS_{14} and ICS_{21} treatments.

The highly significant increase ($P < 0.01$) in OM content was most probably attributable to several factors. First, fermentation may reduce ash and other inorganic components, thereby proportionally increasing the OM fraction of the feed (Rosly and Soon, 2025). Second, microbial growth may contribute additional organic biomass, particularly lipids and carbohydrates, thereby increasing OM content (Siddik et al., 2024; He and Cui, 2025). Third, fermentation promotes the breakdown of complex carbohydrates, fibre fractions, and anti-nutritional compounds, increasing nutrient availability and the proportion of digestible OM (Ling et al*.,* 2022; Khadka et al., 2025). The higher OM, total CHO, and EE contents in ICS_7 were associated with an increased ME value (3 380 cal/kg DM) compared with those recorded in ICS_{14}, ICS_{21}, and ICS_0.

The CP content (19.57–20.78%) recorded in the current study was similar to values reported previously (Ridla et al*.,* 2016; Oematan, 2020; Mullik et al*.,* 2024). The lack of a significant effect of incubation period on CP ($P > 0.05$; Table 1) could be attributed to the relatively high protein content of *C. odorata* (20.78%), which supported microbial growth and compensated for protein losses during fermentation. Moreover, nutrient composition, the C–N ratio (30), and the addition of 1% fresh rumen fluid were identical in all treatments. The comparable physical and chemical conditions in all experimental units may also explain the absence of significant differences in Ca and P concentrations. Although substrate degradation and enzymatic hydrolysis can affect mineral availability and solubility, they are unlikely to substantially alter the total concentrations of these minerals.

The significant reduction in total CF and fibre fractions, followed by a plateau after 14 days of incubation, could be explained by the following three factors. First, although microorganisms can degrade cellulose and haemicellulose, lignin is highly resistant to degradation because of its cross-linked aromatic polymer structure composed of phenylpropanoid units (Van Soest, 1994; Jazi et al*.,* 2019). Consequently, after the initial reduction (first 7–14 days), the remaining fibre fraction is likely to consist largely of lignin-bound material that is less susceptible to microbial degradation. Cellulolytic microorganisms and their enzymes (endoglucanases, exoglucanases, β-glucosidases) hydrolyse cellulose to glucose (Musa and Elnour, 2024; Li et al*.,* 2025b). Cellulose content was significantly lower ($P < 0.01$)

in all biofermented treatments (19.51–20.53%) than in the non-fermented control (23.11%) (Table 2). This result was expected since incubation allowed fermentative microorganisms and hydrolytic enzymes to degrade cellulose and haemicellulose in *C. odorata.* Extending the incubation period to 21 days did not provide any additional benefit in terms of cellulose degradation.

The second factor may be the depletion of readily fermentable substrates. Microorganisms are likely to utilise the most accessible carbohydrates during the early stages of fermentation, thereby reducing substrate availability as incubation progresses. The factor is the accumulation of inhibitory by-products (organic acids and phenolic compounds) that reduce microbial activity. A comprehensive review by Yoon et al. (2024) showed that organic acids such as citric, lactic, propionic, and acetic acids exert antibacterial effects through multiple mechanisms, including osmotic stress, membrane disruption, cytoplasmic acidification, and cytoplasmic enzyme destabilisation. Laboratory studies reported that organic acids can reduce the cell density of various bacterial species, including *Campylobacter* sp., *Cronobacter sakazakii*, *Escherichia coli*, *Listeria monocytogenes*, *Salmonella enterica*, *Typhimurium*, *Staphylococcus aureus*, *Vibrio parahaemolyticus*, *Vibrio vulnificus*, and *Yersinia enterocolitica* by 1.4–6.5 log units (Kim et al., 2016; Tsai et al., 2018; Chhetri et al., 2019; Hwang et al., 2019; Yoon et al., 2021a; Yoon et al., 2021b; Zhou et al., 2025).

The extent of the reductions in both NDF and ADF depends on the degradation of haemicellulose, cellulose, and lignin. Biofermentation reduced NDF by 33.6%, compared with a 21.4% reduction in ADF (Table 2). This difference was attributable to the substantially greater reduction in haemicellulose (52.9%) than in cellulose (29.6%) and lignin (12.5%). The greater reduction in NDF than in ADF can be explained by the extensive degradation of haemicellulose, which is present in NDF but not in ADF (Van Soest, 1994).

In vitro nutrient digestibility

In vitro digestibility reflects the proportion of feed nutrients that can be utilised by livestock. The present study showed that biofermentation significantly affected *i*DMD and *i*OMD. Both variables increased by day 7, declined at day 14, and increased again by day 21 (Table 3). This fluctuation may have been associated with changes in microbial composition during biofermentation. Readily fermentable carbohydrates from palm sugar stimulated the growth of sugar-utilising microbiota during the first 7 days of incubation, causing an intensified substrate degradation and resulting in higher *i*DMD and *i*OMD values.

Extending the biofermentation period to 14 days likely reduced the availability of easily fermentable carbohydrates, resulting in lower growth and activity of microorganisms dependent on these substrates. This decline consequently resulted in lower *i*DMD and *i*OMD values. The degradation of organic compounds during the first 14 days may also have generated substrates that supported the proliferation of microorganisms capable of degrading more complex organic matter. As a result, degradative activity increased again by day 21, which was reflected in the higher *i*DMD and *i*OMD values observed in ICS_{21}. Similar findings were reported by Kurnia et al. (2024), who showed that extending the biofermentation period of *C. odorata* to 21 days significantly improved *i*DMD from 61.60 to 72.55% and *i*DMD from 60.61 to 71.61%. These results are consistent with the concept of microbial succession during biofermentation described by Williams et al. (2017), whereby sugar-utilising microorganisms dominate the early stages, while fibre-degrading microorganisms become more abundant later in the process.

Unlike *i*DMD and *i*OMD, *i*CPD and *i*CLD was not affected by the duration of biofermentation ($P > 0.01$). This outcome may be explained by two plausible factors. First, CP and CL contents were similar in all treatments, providing comparable amounts of substrate for microbial utilisation during biofermentation. Second, the relatively high CP (20.78%) and CL (7.13%) contents of *C. odorata* may have masked any additional contribution of microbial biomass generated during fermentation. These findings differ from those reported by Nomleni et al. (2025), who observed a significant linear decline in *i*CPD (80.03–78.26%) together with a significant increase in *i*CLD (64.24–79.76%) when the incubation period was extended to 21 days. These discrepancies could be related to differences in the carbon sources used during biofermentation. In the present study, palm sugar was employed as a rapidly fermentable carbohydrate, whereas Nomleni et al. (2025)

utilised a moderately fermentable carbohydrate source. Previous research has shown that microorganisms utilising readily available sugars tend to channel carbon into fermentation products rather than lipid biosynthesis (Yao et al., 2020; Jia et al., 2024). In contrast, some fibre-degrading microbes divert carbon to lipid storage or structural fatty acids, thereby providing an energy reserve

for prolonged survival (Williams et al., 2017; Hou et al., 2021; Zhong et al., 2025).

Anti-nutritional compounds

Fermentation, supplementation with exogenous enzymes, dryheat treatments, and autoclaving have been shown to reduce the concentrations of anti-nutritional compounds, although the extent of reduction varies among treatments and types of anti-nutritional compounds (Mulik et al., 2016; Olude et al., 2016; Ridla et al., 2016; Pasha et al., 2020). In general, extending the incubation time up to 21 days (ICS_{21}) in the present study provided greater opportunity for microbial hydrolysis of anti-nutritional compounds, resulting in their lower concentrations. This was in line with an *in vitro* study by Arjmand et al. (2023), who showed that extending fermentation time to 24 h using *Saccharomyces cerevisiae*, *Lacticaseibacillus casei,* and *Lactiplantibacillus plantarum* significantly reduced saponin and phytic acid concentrations by 67.21 and 64.64%, respectively.

Transformation of the data presented in Table 4 into graphical form (Figure 1) revealed four noteworthy patterns. First, even though fermentation period had a highly significant effect ($P < 0.01$) on the concentrations of all four secondary metabolites, the rate and extent of reduction differed between compounds. This phenomenon has also been observed by other authors (Mulik et al., 2016; Olude et al., 2016; Ridla et al., 2016; Pasha et al., 2020; Faizal et al., 2023), who reported that the magnitude of reduction varied among treatments and different anti-nutritional compounds.

Second, phytic acid showed the greatest decrease (51.92%), corresponding to a reduction rate of 2.47% per day (0.742 percentage points per 7-day incubation period). Chemically, phytates are hexaphosphates of myo-inositol and are the major storage form of phosphorus in plants (Banerjee et al., 2023; Choi and Choi, 2025). Since phytates are heat-stable anti-nutritional compounds that bind to Ca^{2+}, Mg^{2+}, Zn^{2+}, Cu3+, and Fe^{3+} ions and prevent their absorption by livestock and humans (Faizal at al., 2023; Choi and Choi, 2025), the reduction in their concentration observed in the present study is unlikely to have resulted from heat generated during biofermentation. A more plausible explanation is the hydrolysis of phytic acids by phytases produced by fermentative microorganisms. Phytases have been identified in bacteria, archaea, and eukaryotes (Nogimori et al., 1991). Among these, *Lactobacillus* spp. are recognised as one of the main phytase producers in feed biofermentation that can degrade phytic acids into inositol and phosphate (Nuobariene et al., 2015; Rollán et al., 2019; Choi and Choi, 2025). The high reduction rate of phytic acid degradation observed in the present study indicates

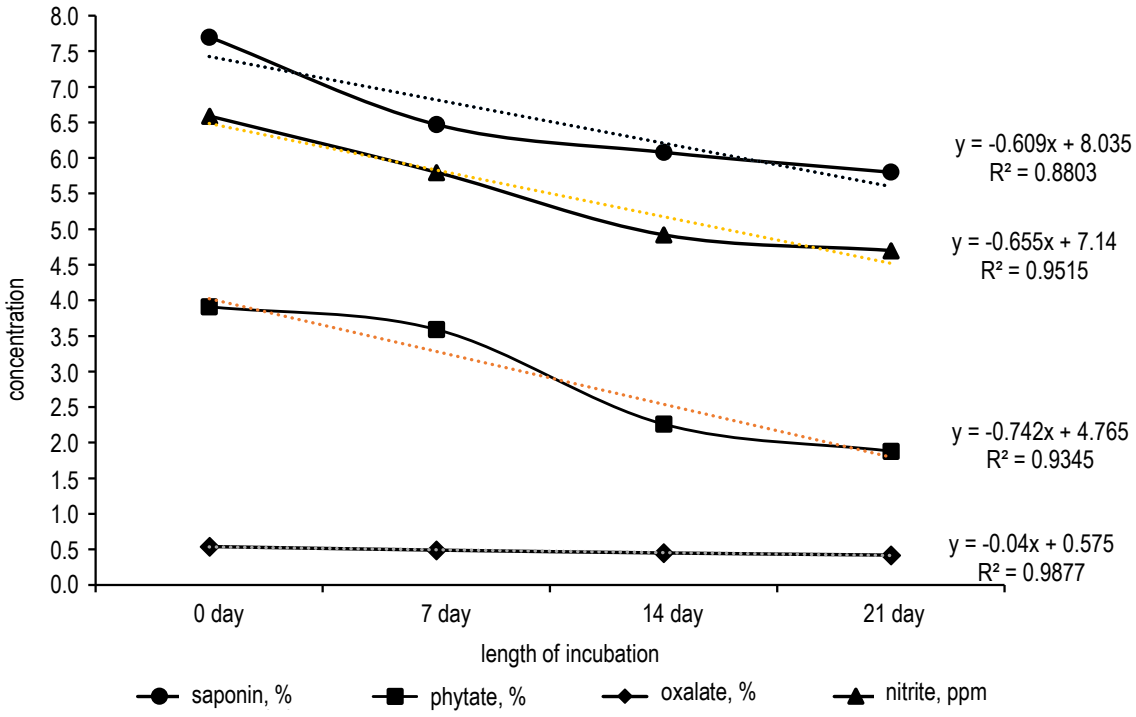

Figure 1. Reduction trend in saponin, phytic acid, oxalate and nitrite concentration in Chromolaena odorata leaves fermented for 0, 7, 14, and 21 days

the presence of phytase-producing microbes in the biofermentation of *C. odorata*. Banarjee et al. (2023) reported that phytic acid content in rice bran was 5.8% and 11.4% in mustard.

Thirdly, the reductions in saponin (24.67%), oxalate (22.22%), and nitrite (28.68%) levels were similar. This finding suggests that although these compounds are degraded through different enzymatic and non-enzymatic pathways, their overall rates of hydrolysis is not significantly different (Samtiya et al*.,* 2020).

The approximately 24.7% reduction in saponin found in the present study (Table 4) most likely originated from a combination of mechanisms involved in biofermentation. According to He et al. (2019), natural saponins can be degraded by heat generated during fermentation, acidolysis, and microbial transformation. Saponins are a group of high-molecular-weight glycosides (1000–1500 Da) consisting of one or more sugar moieties (commonly glucose, galactose, glucuronic acid, xylose, rhamnose, or methyl pentose) linked glycosidically to a hydrophobic glycone (sapogenin) of either triterpenoids or steroids (Gadouche et al*.,* 2023; Ramdani et al., 2023). Microbial degradation of saponin occurs mainly through enzymatic hydrolysis of glycosidic bonds, such as β-glucosidase, β-galactosidase, and α-L-rhamnosidase, resulting in the formation of sapogenins and sugars, which facilitates detoxification and biotransformation (Love and Simons, 2020; da Costa et al*.,* 2024; Li et al*.,* 2024).

Oxalate degradation during feed fermentation is mediated primarily by lactic acid bacteria and specialised oxalate-degrading microorganisms. Key microbes involved in this process include *Oxalobacter formigenes*, a specialist oxalate-degrading bacterium, as well as lactic acid bacteria such as *Lactobacillus*, *Bifidobacterium*, *Lactobacillus fermentum*, and *Lactiplantibacillus plantarum* (Kim et al*.,* 2021; Soliman et al*.,* 2021). Main enzymes that participate in oxalate hydrolysis are oxalyl-CoA decarboxylase, which converts oxalyl-CoA to formyl-CoA and CO_2, and formyl-CoA transferase, which transfers CoA groups, enabling oxalate metabolism (Zhang et al*.,* 2022). However, the mechanisms responsible for oxalate degradation were not investigated in the present study. Therefore, it was not possible to identify the microorganisms or enzymes that contributed to oxalate hydrolysis during *C. odorata* biofermentation.

Nitrite concentrations in feedstuff can be reduced during biofermentation through microbial activity, enzymatic conversion, and, to a lesser extent, the effects of heat. In practice, nitrite is primarily reduced rather than hydrolysed, with microbial nitrite reductases converting nitrite to ammonia or gaseous nitrogen compounds. These three mechanisms were likely involved in the 28.8% reduction of nitrite concentration observed in *C. odorata* in the present study. Microorganisms reported to participate in nitrite reduction include *Pseudomonas*, *Paracoccus, Escherichia coli, Clostridium*, and *Lactobacillus plantarum* (Kung et al., 2018; Majou and Christieans 2018; Zhang et al., 2022; Shen et al*,* 2023). Four types of denitrifying enzymes have been identified in these pathways, i.e. NADH-dependent nitrite reductase (Nir), which converts nitrite to ammonia under fermentative conditions, cytochrome cd1 nitrite reductase (NirS), which reduces nitrite to nitric oxide in denitrifiers, and copper-containing nitrite reductase (NirK), which performs a similar function in other bacterial groups. Nitrite oxidoreductase, although primarily associated with nitrification rather than denitrification, also plays a role in nitrogen cycling (Wang et al., 2019; Ikeyama and Tabe, 2022; Wang et al., 2024). Moderately high temperatures (approximately 30–40 °C) can enhance microbial activity during fermentation, accelerating nitrite decomposition into nitric oxide and nitrogen dioxide, whereas higher temperatures (>50 °C) may reduce microbial viability and limit enzymatic nitrite reduction (Sallan et al*.,* 2020; Jantapirak et al*.,* 2023). Because heat generated during biofermentation is generally moderate, temperature conditions were likely favourable for microbial nitrite conversion in the present study.

Finally, extending the biofermentation period of *C. odorata* beyond 14 days did not result in further significant reductions in the concentrations of the antinutritional compounds (Figure 1). This trend may be attributed to a gradual decline in microbial and chemical activity resulting from the depletion of fermentable substrates and the accumulation of fermentation products. Similar observations have been reported by Saha et al. (2020), who have noted that declines in fermentative microbial activity are common during prolonged biofermentation as the process progresses through hydrolysis, acidogenesis, acetogenesis, interspecies electron transfer, and methanogenesis. In addition, fermentation products such as organic acids, ethanol, and gases may exert negative feedback on microbial metabolism by inhibiting further microbial growth, slowing enzymatic activity, and altering microbial community structure (Jia et al., 2025; Bao et al., 2025). Although this self-limiting effect stabilises fermented substrates, it may also reduce the extent of further substrate degradation (Saha et al., 2020; Wang et al., 2024; Du and Cai, 2025). A significantly

lower organic matter content in all bio-fermented substrates (ICS_7, ICS_{14}, and ICS_{21}; Table 1) supports this argument.

Conclusions

Biofermentation is a low-cost feed pretreatment technique that has been shown to improve the feeding value of *Chromolaena odorata* by enhancing nutrient digestibility, reducing fibre fractions, and lowering concentrations of anti-nutritional compounds. However, one of the remaining challenges is the relatively long fermentation period, which requires a minimum of 21 days. Therefore, this study focused on shortening the incubation time through the use of liquid sugar as a readily available carbon source. The results of this research demonstrate that liquid sugar can effectively reduce the required biofermentation period of *C. odorata* to 7 days while maintaining improvements in nutritional quality.

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Conflict of interests

The Authors declare that there is no conflict of interest.

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