Veterinary Integrative Sciences 2023; 21(2): 273 - 289 DOI; 10.12982/VIS.2023.022

Vet Integr Sci

Veterinary Integrative Sciences

ISSN; 2629-9968 (online)

Website; www.vet.cmu.ac.th/cmvj

Research article

Effect of locally available and commercial preservatives on nutrient content, organic matter digestibility and microbial changes of wet brewers' grain

Yunus Abdu [1, 2,*], Adugna Tolera[1], Ajebu Nurfeta[1] and Barry Bradford[3]

[1] School of Animal and Range Sciences, College of Agriculture, Hawassa University, P.O.Box 5, Hawassa, Ethiopia
[2] Department of Animal Sciences, , College of Agriculture and Environmental Science , Arsi University, P.O.Box 193, Asella, Ethiopia
[3] Departments of Animal Science, Michigan State University, East Lansing, MI, USA

Abstract

The aim of the study was to evaluate the effect of different preservatives on nutrient content, organic matter digestibility (OMD), ammonia nitrogen (NH_3-N), short-chain fatty acid (SCFA), yeast, mold, and lactic acid bacteria (LAB) concentrations of wet brewery grain (WBG). The experiment was undertaken for 28-days using five treatments (T); T1 = no preservative; T2 = 1.75% salt; T3 = 2.5% molasses; T4 = 2.5% Effective Microbial (EM) and T5 = 0.6% formic acid. Data was analyzed using a completely randomized two way factorial design of the mixed procedure of SAS (Version 9.1, 2001). The highest dry matter loss was for WBG stored with T1, while the lowest was for WBG treated with T5. WBG treated with T5 had the highest crude protein content, while those with T1 had the lowest. OMD of WBG treated with T5 was higher than T4. SCFA content of WBG treated with T3 was higher than those T4. WBG stored with T1 and T3 had a higher NH3N content than T4 and T5. WBG stored with T1 had the highest yeast and mold concentration, while WBG treated with T5 had the least. The highest LAB count was recorded for WBG treated with T5, while the least was for T1 and T3. Results has shown that preserving WBG with 0.6% formic acid is found to be more effective in terms of preventing spoilage by inhibiting mold and yeast growth as compared to other preservatives used in this study.

Keywords: Lactic acid bacteria, Mold, Preservatives, Wet brewery grain, Yeast

Corresponding author: Yunus Abdu, Department of Animal Sciences, College of Agriculture and Environmental Science , Arsi University, P.O.Box 193, Asella, Ethiopia, E-mail: abduyunus45@gmail.com

Article history; *received manuscript: 16 November 2022,*
revised manuscript: 28 December2022,
accepted manuscript: 9 February 2023,
published online: 23 February 2023
Academic editor; *Korakot Nganvongpanit*

INTRODUCTION

In Ethiopia, getting feed with less costs and maximizing profitability becomes a great concern for dairy farmers. With the increasing cost of concentrate there is a growing demand for a wide variety of agro-industrial by-products, such as wet brewer's grains (WBG) which is a by-product of brewing (Robertson et al., 2010). The presence of high protein, rumen undegradable protein (RUP) and easily digestible fiber in WBG aid for utilization as feed for ruminants (Bisaria et al., 1997). With the boom in the beer industry in the last few decades in Ethiopia, fresh brewer's grain is becoming a relatively cheaper alternative protein supplement for dairy cattle feeding in Ethiopia, especially near to brewery factory like Hawassa, Gondar, Debre Birhan and their surroundings (Getu et al., 2021). However, the high moisture content of wet brewery grain (80 to 85%) makes the by-product particularly susceptible to microbial growth and subsequent spoilage in a short period of time (7 to 10 days), which hampers its utilization (Stojceska et al., 2008).

The improper storage of the wet brewery grain under normal environmental conditions as commonly adopted on farms resulted a large loss of DM and nutrients, characterized by an unpleasant odor, and even stimulates mold to produce mycotoxins (Asurmendi et al., 2013). However, several methods have been proposed to prolong WBG storage time. These methods includes: several organic acid solutions, microbial inoculants, storage with salt and physical methods of preservation, including oven-drying, freeze-drying, freezing and use of superheated steam (Hatungimana and Erickson, 2019). Factory drying has been the most effective method of preserving WBG. However, owing to the high energy cost, many breweries, especially those in the developing countries can no longer afford this practice (Ikurior, 1995).

Relatively, organic acid additives are an efficient alternative for the storage of wet brewery grain through ensilage process. Especially formic acid, propionic, lactic, acetic, benzoic, and sorbic acids can directly acidify the feed and immediately reduce pH, there by inhibit the activity of undesirable microorganisms, and eventually reduce the nutrient loss of the ensiled feed (Wen et al., 2017). Formic acid and propionic acid have been considered as organic acids silage additives in numerous studies (Souza et al., 2012). Formic acid is the most acidic fermentation inhibitor; which has suitability for the storage of high-moisture raw materials due to a reduction in the loss of dry matter and nutrients to ensure silage quality (Wen et al., 2017). It was found that including either formic acid (0.20 and 0.40 %) or propionic acid (0.40 %) was effective in reducing subsurface deterioration, but had no effect on surface deterioration (Allen and Stevenson, 1975).

Common salt and molasses have also been used successfully for improved preservation of low dry matter and low water-soluble carbohydrate material under anaerobic conditions (Allen and Stevenson, 1975). Hatungimana and Erickson (2019) reported that storing WBG with salt resulted in greater in vitro DM digestibility and in situ DM and protein degradability. The capacity of salt to inhibit mold growth may be mainly explained by its properties of causing plasmolysis and being toxic to molds (Hatungimana and Erickson, 2019). However, it was indicated that use of salt at 1.4% (Anderson et al., 2015) and molasses at 2% (Allen et al., 1975) cannot effectively reduce the

level of mold and yeast, and subsurface deterioration. Effective microbial inoculants (EM), a product characterized by a mix of microorganisms, are also another alternative used to preserve wet brewery grain, because inoculants stimulate fermentation and help to complement the native lactic acid bacteria which maintain a proper fermentation (Muck, 2008).

In Ethiopia, farmers traditionally depend often on either fresh WBG or soaked with brine solution, and seldom on sun drying and ensiling. Moreover, molasses and EM are the cheapest and common ones in Ethiopia. However, no documented information is available on the efficiency of ensiling these local additives with WBG (Getu et al., 2021). Therefore, identifying different preservation methods that are economically feasible and easily applicable to dairy farmers in Ethiopia is very crucial for storing and utilizing this abundant feed without spoilage. However, research done at evaluating locally available substances for preservation of WBG is very scarce and technological applications used by brewery factory and farmers to prevent WBG spoilage limited. The aim of this study was therefore, to evaluate the effect of locally available commercial preservatives (common salt, molasses, EM and formic acid) on nutrient status, organic matter digestibility, NH_3-N, SCFA, yeast, mold and lactic acid bacteria concentrations of WBG.

MATERIALS AND METHODS

Experimental locations, design and Treatments

The study was conducted at Hawassa University, College of Agriculture, School of Animal and Range Science, Hawassa, Ethiopia. The experiment was undertaken for 28-days in completely randomized factorial designs. The microbial study was undertaken in 5 x 4 factorial designs with five treatments and four durations of ensiling (storage) period (7, 14, 21 and 28 days). Whereas, the nutrient composition and in-vitro gas parameters experimentation was undertaken in 5 x 2 factorial designs with five treatments and two storage durations (7 and 28 days).

On day zero of the study, approximately 300 kg of wet brewer's grain (WBG) was delivered to the Research site from Hawassa BGI brewery factory. The WBG was then divided, by using a portable weigh balance, into 20 groups (each having 15 kg weights). Then commercial preservative (common salt, molasses, EM and formic acid) was added on a fresh weight basis randomly to the WBG at a rate of: - no preservatives (control); 1.75% salt; 2.5% molasses, 2.5% EM and 0.6% formic acid. Then treatment (preservatives + WBG) was allowed to mix well using a shovel for 5 min, and then stored in closed plastic containers for 28 days inside a room. The experimental treatments arrangements were: T1 (Control): 15kg WBG without preservative; T2) 0.26 kg Salt + 15kg WBG; T3) 0.38 kg Molasses + 15kg WBG; T4) 0.38 kg EM + 15kg WBG; and T5) 0.09 liter formic + 15kg WBG

Sampling and Measurements

Samples weighing approximately 200 g were taken from each separate plastic container twice a week, beginning at day 0 and ending at day 28. The two consecutive samples collected within a week were merged (bulked) at

the end of each week, and representative subsample were taken for analysis, and the remaining samples were discarded. Samples for chemical and in-vitro gas production were analyzed on days 7 and 28, while sample for microbial analysis and pH were analyzed on day 7, 14, 21 and 28. Samples were collected from the center to different corners of the container to avoid sampling in the same spot consecutively. Sampling spots were always different from previous ones. Samples were then refrigerated at 4°C until used for analysis (Allen and Stevenson, 1975; Johnson and Huber, 1987). Samples were analyzed for chemical composition (DM, CP, NDF, ADF, ash, and lignin), in in-vitro gas production variables (ME, OMD, SCFA and NH_3N), presence of molds, yeast and lactic acid bacteria colony and pH.

To determine the total DM loss occurred throughout the storage period, the initial weights at day zero and the final weights of the material remaining in each of the treatment plastic containers was recorded at the end of the 28-day storage period using plastic buckets. Weights of sample taken at each sampling was recorded throughout the study and included in the calculation. Dry matter losses were calculated as a difference of DM for initial fresh WBG and the final weights plus samples that were taken during conservation. The pH during the storage of the fresh WBG was measured using a digital bench top pH meter (Hannan Instrument, pH 210, microprocessor pH meter), where 100 mL of distilled water was added to 10 gram of the sample, according to the method of Cherney and Cherney (2003), and was left to stand for 1 hour before reading.

Yeast and mold count (YMC) was determined by AOAC method 997.02 (AOAC, 1999). A 1:10 dilution of each test portion was aseptically prepared by homogenizing 25g of the sample with 225 ml saline peptone water in a stomacher bag for 30 second. Then a serial dilution of 10^{-1} to 10^{-5} was prepared. All plating was done by the pour plate method. One mL (1 mL) aliquot of each serial dilution was pipetted into sterile petri dishes having a label corresponding to the dilution, and then 15 mL of molten potato dextrose agar medium with chloramphenicol and streptomycin (to restrict bacterial growth) was added to cover the base of the petri dishes. The petri dishes was swirled clockwise and anticlockwise to ensure uniform mixing and allowed to set. Then gelling agent was allowed to solidify and then plates were incubated at 25±1°C for 5 days. The total number of yeast and mold colonies per plates was calculated by multiplying number of yeast and mold colony counted with dilution factor. Then the final results were reported as colony-forming units/gram (CFU/g). Similarly, lactic acid bacteria were quantified by using MRS agar (De Man, Rogosa and Sharpe) after incubation in an oven for 48 hours at 37 °C.

Gas production (GP) was determined according to Menke and Steingass (1988). About 200 mg of sample was weighed and transferred into 100 ml calibrated glass syringes, fitted with white vaseline-lubricated glass plungers. A buffer solution was prepared and maintained in a water bath at 39 °C under continuous flushing with CO_2. Rumen fluid was collected from the rumen of sheep soon after slaughter from abattoir. The rumen fluid was strained through four layers of cheese cloth, and then was mixed, filtered and added to the buffer solution (1:2 v/v) under constant stirring at 39 °C and under oxygen-free CO_2. Thirty mL of buffered rumen fluid was injected into each syringe, which was then immediately placed into a rotating disc and oven-incubated at constant temperature of 39 °C. Three syringes with only buffered rumen fluid, termed as blanks, plus 3 syringes with each treatment standard with known GP was

included in each run. GP of the samples, blanks and standards was recorded at 0, 3, 6, 9, 12, 24, 32, 48, 72 and 96 hours of incubation. The plunger of the syringe was re-set to 30 ml after the 6 and 24 hour readings.

For organic matter digestibility (OMD), metabolizable energy (ME) and short chain fatty acid (SCFA) estimation, the GP of the feed samples was recalculated as 24 h GP on 200 mg DM using results from the blanks, with the corrections determined by the standards of hay and concentrate, the sample weight and its DM concentration. The estimations of OMD and ME were carried out according to Menke and Steingass (1988) and short-chain fatty acid (SCFA) production according to Getachew et al. (2002) by using the following equations:

$$ME\ (MJ/kg\ DM) = 2{:}20 + (0.136 * GP_{24}) + (0{:}057 * CP)$$
$$OMD\ (\%) = 14{:}88 + (0{:}889 * GP_{24}) + (0{:}45 * CP) + (0{:}651 * XA)$$
$$SCFA\ (mmol) = (0{:}0222 * GP_{24}) - 0{:}00425$$

Where GP, CP and XA are corrected 24 h gas volume (ml/200 mg), crude protein (%DM) and ash (%DM) of the incubated samples, respectively.

Ammonia nitrogen (NH_3-N) concentration in fermentation liquid of each syringe was determined according to Karabulut et al. (2007) using Kjeldahl method. NH_3-N was determined after 96 h incubation in the supernatant fraction by steam distillation; 2 ml 1 M NaOH was added to 5 ml of the supernatant fraction diluted with 30 ml water and the solution was directly distilled and ammonia-N evolved was collected into boric acid (30 g/L). Then, the distillate was titrated with 0.05 M H_2SO_4.

Chemical analysis

Partially dried ground samples were analyzed for DM, total ash, CP, and EE using the procedure described by AOAC (1990). The DM content of the samples was determined by drying the sub-samples at 105°C in oven for 24 hours. The total ash content of samples was determined by igniting the dry samples at 600°C for 5 hours in a muffle furnace. The total N content of samples was determined using the Kejldhal procedure and the crude protein was computed as CP = N × 6.25. The EE was determined by heating the dry sample with petroleum ether for 8 hours. The NDF and ADF concentration was sequentially determined using an ANKOM 200 fiber extractor (ANKOM Technologies, Fairport, NY, USA) according to the method of Van Soest and Robertson (1985).

Statistical Analysis

All data were subjected to analysis of variance using a completely randomized two way factorial design of the general linear model (GLM) procedure of SAS (Version 9.1, 2001). Fixed effects include treatments (factor A) and storage duration (factor B). Mold, yeast and lactic acid bacteria count (colony-forming units (CFU) per gram) were logarithmically transformed for inclusion in the model. Results were expressed as least squares means with the lowest standard error. Significance was declared at $P < 0.05$. Data was analyzed according to the following model:

$Yijk = \mu + Ti + Dj + (TD)ij + \varepsilon ijk$

Where:

yijk = observation k in level i of treatments (factor A) and storage duration (factor B)

μ= the overall mean

Ti = effect of level treatments (i = 1, 2, 3, 4, 5)

Dj = effect of storage duration (j = 4 for microbial analysis, or j = 2 for other parameters)

(TD) ij = effect of interaction between level i of treatment and level j of storage duration

εijk = random error

RESULTS

Chemical composition

The average chemical composition of wet brewer's grain stored using five treatments over the 28 days period is presented in Table 1. There were significant differences among treatments for all parameters measured. The DM, NDF, ash and lignin content of WBG were significantly affected by the interaction of treatment and storage durations. In contrast, the CP and ADF content of WBG were not affected by the interaction of treatment and storage durations. Higher mean DM content was recorded for WBG treated with 0.6% formic acid and 1.75% salt, while the lowest was for WBG stored with no preservatives and 2.5% EM. The mean CP content of wet brewer's grain stored with 0.6% formic acid was the highest, while those stored with no preservatives was the lowest during the 28 days of storage. Relatively, wet brewer's grain treated with 1.75% salt and 2.5% EM had similar CP content, which was higher than wet brewer's grain treated with 2.5% molasses and no preservatives. The mean NDF content of WBG stored with no preservatives were higher than the NDF content all WBG treated with preservatives. The average ash and lignin content for WBG treated with 1.75% salt and 2.5% molasses was higher than ash and lignin content of WBG treated with 0.6% formic acid. The higher DM loss was for WBG stored with no preservatives, while the lowest was for WBG treated with 0.6% formic acid.

Table 1 Effects of treatment, storage duration and interaction of treatment and storage duration on chemical composition of wet brewer's grain stored for 28 days

Parameters (g/kg DM)	Treatment					Duration		*P*		SEM	*P*
	Control	1.75% Salt	2.5% Mol	2.5% EM	0.6% FoA	7	28	T	D	T x D	T x D
DM	286.4^{c}	294.8^{a}	290.2^{b}	285.4^{c}	296.8^{a}	287.8^{b}	293.6^{a}	***	***	1.27	**
CP	233.9^{d}	247.7^{b}	239.8^{c}	247.1^{b}	260.8^{a}	246.7	245.1	***	NS	2.68	NS
NDF	581.0^{a}	539.1^{b}	544.2^{b}	544.8^{b}	553.9^{b}	614.2^{a}	491.3^{b}	*	**	9.58	*
ADF	221.8	207.8	218	218.7	220.8	225.6^{a}	209.3^{b}	NS	**	5.45	NS
Ash	41.0^{ab}	42.3^{a}	43.3^{a}	41.3^{ab}	39.3^{b}	38.9^{b}	43.9^{a}	**	**	1.00	**
Lignin	84.2^{ab}	85.3^{a}	86.4^{a}	84.4^{ab}	82.3^{b}	81.9^{b}	87.1^{a}	*	**	1.23	*
DML(g)	122.3^{a}	104^{d}	115.5^{b}	110^{c}	96.4^{e}	27.4^{b}	191.9^{a}	***	***	2.35	***

$^{a-e}$ Means within rows with different superscript are significantly different at *P<0.05; ** (P<0.01); *** (P<0.001) for treatment effect.

$^{a-b}$ Means with different superscript within rows are significantly different at (P<0.01); *** (P<0.001) for storage duration effect.

Abbreviation: DM = dry matter, CP = crude protein, NDF = Neutral detergent fiber, ADF = acid detergent, DML = dry matter loss, Control = no preservatives, 2.5% Mol =2.5% Molasses, 2.5% EM = Effective microbial, 0.6% FoA= 0.6% Formic acid; NS = No Significant Difference; SEM = Standard error of mean; T = treatment, D= Storage duration; T x D = treatment by storage duration interaction.

OMD, ME, SCFA and NH_3-N

Effects of treatments (preservatives) on OMD, ME, SCFA and NH_3-N of wet brewer's grain stored for 28 days is presented in Table 2. The OMD, ME and SCFA content of wet brewery grain were not affected by the interaction of treatment and storage durations. Storage durations also did not affected the OMD, ME and SCFA content of wet brewery grain, but treatment did affect significantly ($P < 0.05$) the OMD, ME and SCFA content. The mean OMD, ME and SCFA content of WBG treated with 0.6% formic acid and 2.5% molasses were higher than those WBG treated with 2.5% EM and no preservatives. On other hand, the NH_3N content of WBG was significantly affected by the interaction of treatment and storage durations. The WBG with no preservatives had a higher NH_3N content than WBG treated with 2.5% EM and 0.6% formic acid during the 28 days of storage time. Relatively, the NH_3N content of WBG stored with no preservatives was similar with WBG treated with 2.5% molasses and 1.75% salt.

Table 2 Effects of treatment, storage duration and interaction of treatment and storage duration on organic matter digestibility, metabolizable energy, short-chain fatty acid and ammonia nitrogen content of wet brewer's grain stored for 28 days

Parameters (g/kg DM)	Treatment					Duration		*P*		SEM	*P*
	Control	1.75% Salt	2.5% Mol	2.5% EM	0.6% FoA	7	28	T	D	T x D	T x D
OMD (%)	64.9^{ab}	63.9^{b}	65.2^{ab}	63.8^{b}	66.6^{a}	64.9	64.9	*	NS	0.95	NS
ME (MJ/Kg DM)	9.17^{ab}	8.99^{b}	9.19^{ab}	8.98^{b}	9.42^{a}	9.17	9.13	*	NS	0.15	NS
SCFA (mmol)	0.92^{ab}	0.87^{b}	0.91^{ab}	0.87^{b}	0.93^{a}	0.90	0.92	*	NS	0.02	NS
NH3-N (mg/kg)	4.02^{a}	3.76^{ab}	3.81^{ab}	3. 56^{b}	3.11^{c}	3.40^{b}	3.91^{a}	***	***	0.15	**

$^{a-c}$ Means within rows with different superscript are significantly different at * (P<0.05); *** (P<0.001) for treatment effect.

$^{a-b}$ Means with different superscript within rows are significantly different at *** (P<0.001) for duration effect.

Abbreviation: OMD = Organic matter digestibility, ME = Metabolizable energy, SCFA= Short chain fatty acid, NH_3-N = Ammonia nitrogen, Control = no preservatives, 2.5% Mol =2.5% Molasses, 2.5% EM = Effective microbial, 0.6% FoA= 0.6% Formic acid, NS = No Significant Difference; SEM = Standard error of mean; T = treatment, D= Storage duration; T x D = treatment by storage duration interaction.

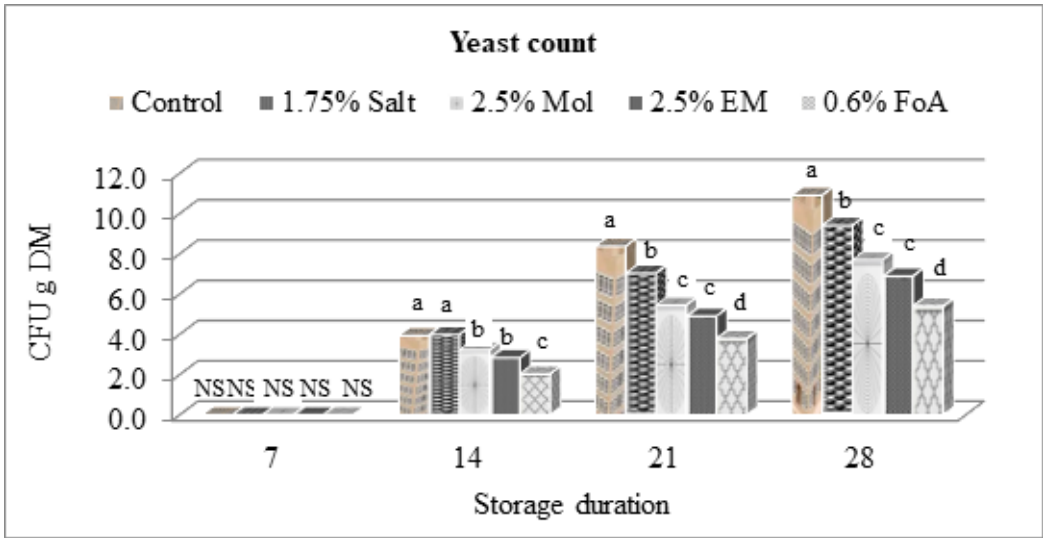
Yeast, mold growth and lactic acid bacteria, and pH.

Effects of treatments on yeast, mold and lactic acid bacteria counts of wet brewer's grain stored for 28 days is presented in Table 3. The yeast and mold count of WBG were significantly affected ($P < 0.05$) by the interaction of treatment and storage durations. However, the yeast and mold count of WBG was unaffected by treatments ($P > 0.05$) during the first seven and 14 days of storage times, respectively (Table 3 and Figure 1). The mean yeast and mold concentration of WBG stored with no preservatives was the highest ($P<0.05$), while the lowest was for WBG treated with 0.6% formic acid (Table 3). However, the mold concentration of WBG treated with 2.5% molasses and 2.5% EM were similar with those treated with 0.6% formic acid ($P > 0.05$) at 21 days of storage. Relatively, at 28 days of storage, there was no variation in mold count between WBG stored with no preservatives and 1.75% salt. The greater yeast and mold concentration in WBG sample was recorded at 28 days of storage, while the least was recorded at 7 days of storage for yeast and at 7 and 14 days of storage for mold (Figure 1).

Table 3 Effects of treatment, storage duration and interaction of treatment and storage duration on yeast, mold and lactic acid bacteria count (log CFU/g WBG), and pH of brewer's grain stored for 28 days.

Parameters	Treatment					Duration				*P*		SEM	*P*
	Control	1.75% Salt	2.5% Mol	2.5% EM	0.6% FoA	7	14	21	28	T	D	T x D	T x D
Yeast	5.82^{a}	5.10^{b}	4.09^{c}	3.66^{d}	2.80^{e}	0.00 d	3.20^{c}	5.90^{b}	8.07^{a}	***	***	0.27	***
Mold	1.25^{a}	1.11^{b}	1.02^{c}	0.96^{d}	0.79^{e}	0.00^{c}	0.00^{c}	1.36^{b}	2.75^{a}	***	***	0.03	***
LAB	3.48^{c}	3.71b^{c}	3.58^{c}	3.94^{b}	4.24^{a}	3.08^{c}	4.01^{b}	5.95^{a}	2.08^{d}	***	***	0.20	NS
pH	4.21^{a}	4.12^{b}	3.90^{c}	4.06^{b}	3.94^{c}	4.83^{a}	4.10^{b}	3.7^{c}	3.55^{d}	***	***	0.06	NS

$^{a-e}$ Means within rows with different superscript are significantly different at *** ($P<0.001$) for treatment effect.
$^{a-d}$ Means with different superscript within rows are significantly different at *** ($P<0.001$) for duration effect.
Abbreviation: LAB = lactic acid bacteria, CFU/g = colony forming unit per gram wet brewery grain, Control = no preservatives, 2.5% Mol =2.5% Molasses, 2.5% EM = Effective microbial, 0.6% FoA= 0.6% Formic acid, NS = No Significant Difference; SEM = Standard error of mean; T = treatment, D= Storage duration; T x D = treatment by storage duration interaction.



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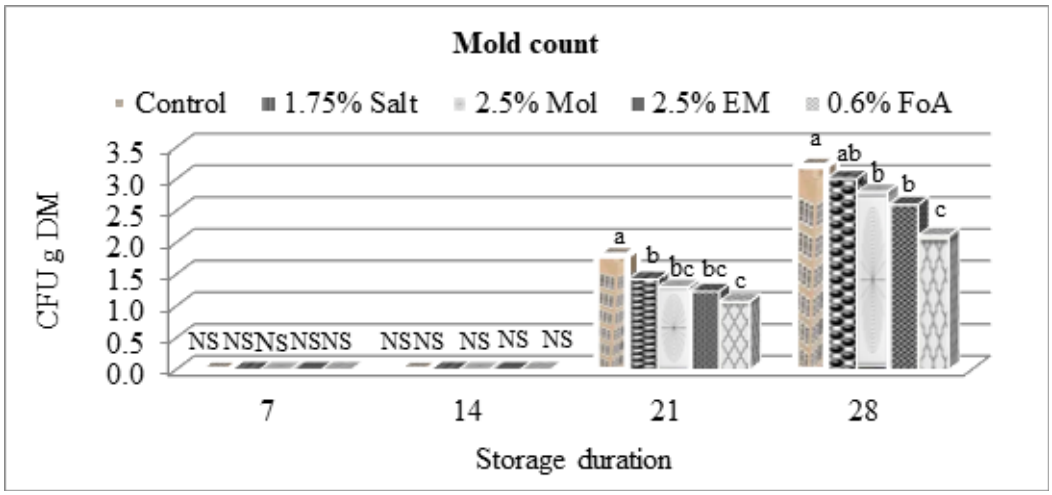
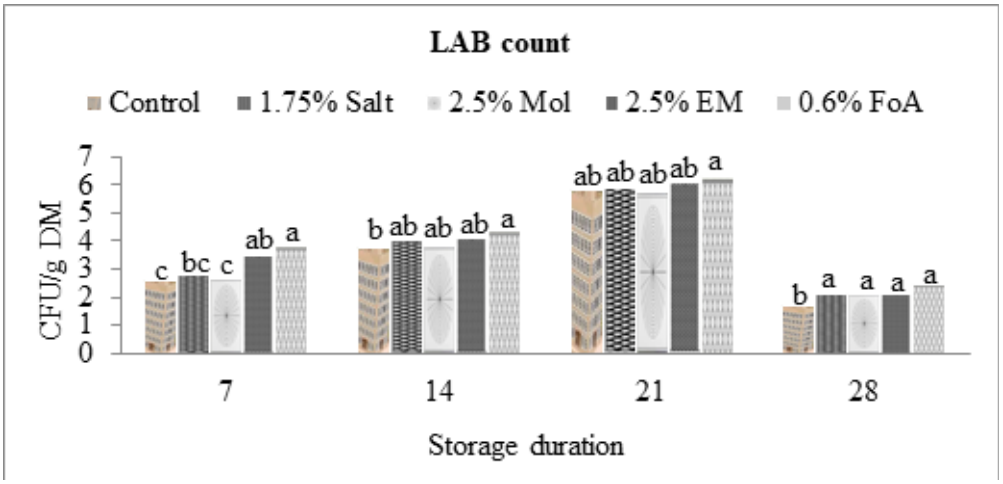


B

Figure 1 (a, b). Effect of treatment and storage duration (days) on yeast and mold count (CFU/g DM) of brewer's grain. [a,b,c] Values are significantly different ($P < 0.05$); Control = no preservatives; 2.5% Mol = 2.5% Molasses; 2.5% EM = 2.5% Effective microbial; 0.6% FoA= 0.6% Formic acid. CFU/g DM = colony forming unit per gram dry matter

On other hand, the LAB count and pH of WBG were not affected by the interaction of treatment and storage durations. WBG treated with 0.6% formic acid had a higher average LAB count than those WBG stored with no preservatives and with other preservative as well (Table 3). Relatively, the LAB count at 21 days of storage was higher for WBG treated with 0.6% formic acid than those WBG treated with 2.5% molasses. At 28 days of storage the LAB count in WBG treated with 0.6% formic acid were higher than those WBG stored with no preservatives. However, the LAB count in WBG treated with different preservatives was similar at 28 days of storage. The highest LAB count was recorded at 21 days of storage, while the lowest was recorded at 28 days of storage (Figure 2).



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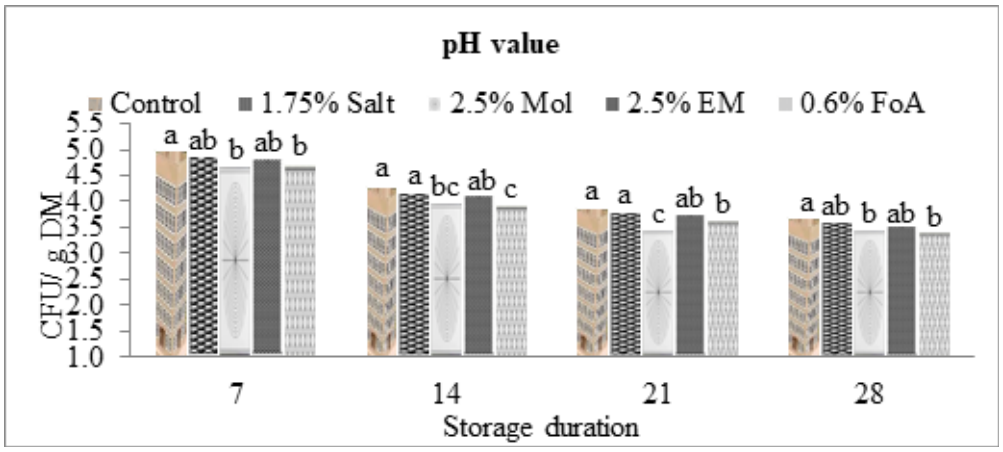

B

Figure 1 (a, b). Effects of treatment and storage duration (days) on lactic acid bacteria count (CFU/g DM) and pH of brewer's grain. [a,b,c] Values are significantly different ($P < 0.05$); Control = no preservatives; 2.5% Mol = 2.5% Molasses; 2.5% EM = 2.5% Effective microbial; 0.6% FoA = 0.6% Formic acid; CFU/g DM = colony forming unit per gram dry matter

The pH of WBG was unaffected by interaction effects of storage durations and treatment. The mean pH of WBG stored with no preservatives was the highest, while the pH of WBG treated with 0.6% formic acid and 2.5% molasses were the least (Table 3). Relatively, at 14 and 21 days of storage time, the WBG with no preservatives and treated with 1.75% salt had a higher pH value than WBG treated with 0.6% formic acid and 2.5% molasses. The highest pH ($P < 0.05$) was recorded at 7 days of storage, while the lowest was recorded at 28 days of storage (Figure 2).

Table 4 In vitro gas production of treatments, storage durations and their interaction effect during the 96 hour (hr) of measurements

Treatments	3hr	6 hr	9 hr	12 hr	24 hr	48 hr	72 hr	96 hr
1	32.7	34.8^{a}	36.8^{a}	38.9^{ab}	41.2	41.4^{ab}	41.1	39.9
2	32.8	34.2^{ab}	36.1^{ab}	37.5^{bc}	38.3	39.5^{b}	37.9	36.9
3	32.3	34.3^{ab}	36.3^{ab}	38.1^{ab}	40.3	41.3^{ab}	40.4	39.3
4	31.8	33.4^{b}	35.0^{b}	37.2^{c}	39.4	39.5^{b}	39.8	38.5
5	32.3	35.2^{a}	37.5^{a}	39.7^{a}	42.1	42.1^{a}	41.7	40.5
SEM	0.29	0.37	0.49	0.55	1.02	0.76	1.03	1.06
P-T	NS	*	*	**	NS	*	NS	NS
7d	32.3	34.4	36.8^{a}	38.5^{a}	40.2	40.9	40.4	39.2
28d	32.4	34.3	35.9^{b}	37.6^{a}	40.3	40.7	39.9	38.8
SEM	0.19	0.23	0.31	0.35	0.65	0.48	0.65	0.67
P-D	NS	NS	*	*	NS	NS	NS	NS
SEM (txd)	0.42	0.52	0.69	0.78	1.45	1.07	1.46	1.50
P(txd)	**	**	**	**	NS	NS	NS	NS

$^{a-c}$ Means with different superscript within rows are significantly different at *(P<0.05); ** (P<0.01) treatment effect.
$^{a-b}$ Duration means with different superscript within rows are significantly different at *(P<0.05) for duration effect.
Abbreviation: NS = No Significant Difference; SEM = Standard error of mean; T = treatment, D= Storage duration; T x D = treatment by storage duration interaction

DISCUSSION

Chemical composition

The higher DM content observed for WBG treated with 0.6% formic acid at 28 days of storage (Table 1) could be attributed to the ability of formic acid to reduce the loss of DM, which may be due to the antibacterial property of formic acid against harmful bacteria and the ability of formic acid to rapidly reduce pH. In agreement with the findings, Jingyi et al. (2020) reported that WBG stored with formic acid had the highest contents of DM as compared to WBG treated with propionic acid and control. Relatively, the higher DM content of WBG stored with 1.75% salt could partially be associated to the lessening of water activity by salt, which likely cause microbial and enzymatic processes to be interrupted (Albarracin et al., 2011). Sodium chloride is also regarded as "fermentation inhibitor" in feed preservation process (McDonald et al., 1991).

The lower DM content observed for WBG stored without preservatives (Table 1) was most probably due to the more DM loss occurred as result of the excess gases produced by large population of yeasts, which had developed in the residue during storage period (Souza et al., 2012). According to McDonald et al. (1991), a large population of epiphytic yeasts converts the carbohydrates into ethanol, CO2 and water, which causes an excessive loss of DM. However, the lower DM content observed in WBG treated with 2.5% EM at 28 days of storage was not expected.

The lower DM loss observed in WBG treated with 0.6% formic acid and 1.75% salt in the study was a good sign of the absence of any significant degradation of nutrients during the ensiling process. In contrast, the higher DM losses observed in WBG without preservatives in the present study indicated that the soluble nutrients could have been degraded, due to the large numbers

of yeasts and molds. Nadeau et al. (2000) indicated that the proliferation of harmful microorganisms decomposes protein, sugar, and hemicellulose, which significantly increase the loss of DM, NDF, and water soluble carbohydrate content.

The higher mean CP value observed for WBG treated with 0.6% formic acid indicate that a lower proteolysis might have occurred in WBG treated with formic acid during storage, due to the prevalence of homofermentative microorganisms, which are characterized by a faster rate of fermentation with less proteolysis (Souza et al., 2012). Relatively, the lower mean CP content observed for WBG conserved using no preservatives could probably be associated to the extensive proteolysis that might have occurred during storage. According to Jingyi et al. (2020), a reduction in CP value could be attributed to ammonia losses from proteolysis by increased mold and yeast populations during fermentation.

The higher NDF content recorded for WBG without preservatives as compared to WBG treated with preservatives (Table 1) indicated that the soluble nutrients in WBG stored with no preservatives have been degraded, as a result the proportion of fiber components tended to have proportionally increased (Getu et al., 2021). The nutrient composition of WBG observed in this study was similar to the results reported by Jingyi et al. (2020) and Getu et al. (2021). However, differences in WBG composition reported in many literature sources, and this variation could be related to the differences in the amount and type of grain used during the brewing process (Ferraretto et al., 2018).

The nutritional compositions of WBG, except for CP and ADF, were significantly affected by the interaction of treatment and storage durations (Table 1), as a result the NDF and ADF content were decreased, while the DM, ash and lignin increased with increasing storage duration ($P < 0.05$). The constant increase of ash and lignin, on the opposite trend to ADF and NDF, indicating that the loss of the cell wall components occurred from the WBG as storage periods were advancing. In agreement with the finding, report indicated that the increased ash and lignin contents of WBG during storage could be due to the relative decrease in the other cell contents (Santos et al., 2010) or loss in organic matter that proportionally increased the ash content (Getu et al., 2021).

OMD, ME, SCFA and NH_3-N

The similar in vitro OMD, ME and SCFA content observed across WBG stored with and without preservative implied that adding preservatives could not improve OMD, ME and SCFA content as compared to WBG stored with no preservatives. However, adding 0.6% formic acid could possibly enhanced OMD, ME and SCFA of WBG as compared to 2.5% EM and 1.75% salt (Table 2). This was possibly due to the lesser DM loss of WBG treated with 0.6% formic acid. In agreement with this finding, Wen et al. (2017) implied that ensiling WBG with formic acid result a reduction in the loss of dry matter and nutrients, which ensure silage quality. In contrast, the lower OMD and ME content of WBG treated with 2.5% EM and 1.75% salt as compared to those treated with 0.6% formic acid can be possibly linked to the loss of organic matter and other nutrients in stored WBG (Marston et al., 2009).

The higher NH_3-N content observed in WBG stored with no preservatives and with 2.5% molasses as compared to WBG treated with 0.6% formic acid (Table 2) suggests that the protein and amino acids had undergone degradation in WBG without preservatives and treated with 2.5% molasses, due to the proliferation of harmful microorganisms that decomposes protein (Allen and Stevenson, 1975). On other hand, the lower NH_3-N content observed in WBG stored with 0.6% formic acid indicated that the acidic effect of formic acid could be used for maintaining quality of WBG during 28 days of storage. It was indicated that the lower pH in the formic acid group inhibited the growth of harmful bacteria, thus causing this group to have the lowest levels of ammonia-N (Jingyi et al., 2020). However, the concentration of NH_3-N increased across all treatment at an average range from 3.4 to 4.0 mg/kg of DM from day 7 to 28. In agreement with the result, Allen and Stevenson (1975) reported that the protein and amino acids had undergone some degradation as the storage days increased, due to the proliferation of microorganisms that decomposes protein.

Yeast, mold growth and lactic acid bacteria, and pH.

The higher yeasts concentration observed for WBG stored with no preservatives during the 28 days of storage period (Table 3, Figure 1) could possibly reflect the deterioration of the wet brewery grain, which is usually manifested by a change in the odour and loss of dry matter (Souza et al. 2012). Relatively, the least yeast and mold count observed in WBG treated with 0.6% formic acid indicate that the effectiveness of formic acid against both mold and yeast growth in WBG, which may be explained by its ability of lowering the pH and antifungal agents in treated WBG (Marston et al., 2009). This feature makes formic acid more suitable for the storage of low dry matter and low sugar content of raw materials (Nadeau et al., 2000).

On other hand, the lower mean yeast count observed in WBG treated with 2.5% molasses and 2.5% EM as compared to WBG treated with 1.75% salt indicated that 2.5% molasses and 2.5% EM treatment could inhibit yeast more effectively than 1.75% salt treatment (Table 3, Figure 1). This could be due to the different organic acids such as benzoic and sorbic acids presented in molasses and EM having strong antifungal properties (Kleinschmit et al., 2005), by releasing of hydrogen in the cytoplasm and reducing of the pH, which in turn causes the cell to reduce or stop growing (Lambert and Stratford, 1999). Relatively, the lack of salt impacting yeast count in this study was probably due to the tolerance of yeasts to high concentrations of salt (Masui et al., 1979).

In contrast result has indicated that WBG treated with 1.75% salt had similar efficacy as both molasses and EM in preventing mold (Table 3, Figure 1b). In agreement with the present findings, Hatungimana and Erickson (2019) reported that the mold concentration in WBG treated with salt was not different from WBG treated with PRES inoculants. The capacity of salt to inhibit mold growth may be mainly explained by its properties of causing plasmolysis and being toxic to molds, and also lessening of water activity has been viewed as the most likely cause for microbial growth inhibition by salt (Albarracin et al., 2011). However, the growth of mold observed after 21 days of storage (Figure 1b) in all WBG treatmnet could be as a consequence of exposure to the air during sampling of WBG throughout the storage periods. It was reported that the presence of oxygen in the silage mass promotes the action of spoilage

microorganisms and the reduction of soluble sugars and organic acids (Souza et al., 2012). In general, the lowest yeast and mold growth count (log cfu) recorded for WBG treated with preservatives as compared to control (Table 3) suggested that the importance of using preservatives for safe storage of WBG.

The lower average LAB count obtained in WBG stored with no preservatives and 2.5% molasses as compared to WBG treated with 0.6% formic acid and 2.5% EM during 28 days of storage respectively (Table, 3), suggested that a decrease in number of LAB and production of lactic acid, which may be due to a deficiency of carbohydrate (Allen and Stevenson (1975). The higher LAB recorded for WBG treated with 0.6% formic acid in the study indicated that lactic acid bacteria can continue growing in the presence of formic acid for a certain period of time (Jingyi et al., 2020). However, studies have shown that the mean LAB population (3.78 log cfu / g) observed in this study fall within a good range reported for WBG preservation by Driehuis et al. (2001) who observed initial populations of LAB between 3.7 and 6.3 cfu log /g in various materials, without compromising the conservation of the material.

The pH concentration of wet brewers grains treated with preservatives had significantly lower than the control during the 28 days of storage (Figure 2a), evidencing a proper fermentation pattern in WBG treated with preservatives. Relatively, the lower pH observed in WBG treated with 0.6% formic acid and 2.5% molasses as compared to the pH of WBG stored with no preservatives and with 1.75% salt at 14 and 21 days of storage may be related to the acidic properties of 0.6% formic acid and the acidic nature of the organic acid found in 2.5% molasses used. Consistent with the current findings, Jingyi et al. (2020) reported that addition of formic acid significantly decreased the pH compared with the corresponding values in the control and propionic acid treated groups. It was pointed out that formic acid has a lower dissociation constant, Ka, which is the reason why formic acid can induce a more pronounced significant reduction in the pH value of the culture medium and feed crops (Albarracin et al., 2011).

On other hand, an increase in the pH value observed in the WBG stored with no preservatives could be due to the possible proliferation of undesirable microorganisms. According to Guerra et al. (2005), the increase in pH can promote the proliferation of undesirable microorganisms (fungi). Therefore, a pH values less than and equal to 4 for silages having dry matter content greater than 20% considered ideal because it is indicative of the possible inhibition of undesirable microorganisms responsible for secondary fermentation, provided that anaerobic conditions are maintained throughout the storage period. The pH recorded in WBG treated with preservatives after 15 days (Figure 2b); however, could not prevent the development of filamentous fungi and yeasts, because yeasts can even develop equally in 2.0-pH environments (McDonald et al., 1991). In general, the reduction in pH value observed as the hour of recording increased in all the groups in the present study agrees with the finding of Ogbuagu and Ayo (2023).

CONCLUSIONS

The study has shown that WBG stored with preservative slows down growth of yeast and mold development as compared to control. More

interestingly, preserving WBG with 0.6% formic acid was found to be more effective in terms of preventing spoilage by inhibiting mold and yeast growth, and also to preserve nutrient quality and digestibility of WBG as compared to other preservatives used in this study. Future research should investigate the effect of WBG treated with formic acid on in vivo nutrient digestibility and animal performance.

ACKNOWLEDGEMENTS

This work was funded in part by the United States Agency for International Development (USAID) Bureau for Food Security under Agreement # AID-OAA-L-15-00003 as part of Feed the Future Innovation Lab for Livestock Systems.

AUTHOR CONTRIBUTIONS

Corresponding author participated in the collection, analysis and interpretation of data, as well as the writing of the manuscript. All authors have participated in the conception and design of this work, in the research report editing and reviewing of the article, and in the decision to submit the article for publication.

CONFLICT OF INTEREST

The authors declare that they have no known competing interests that could have appeared to influence the work reported in this paper.

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How to cite this article;

Yunus Abdu, Adugna Tolera, Ajebu Nurfeta and Barry Bradford. Effect of locally available and commercial preservatives on nutrient content, organic matter digestibility and microbial changes of wet brewers' grain. Veterinary Integrative Sciences. 2023; 21(2): 273 - 289.