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The Ancestral Use of Ashes in Red Sorghum Malting, an Efficient Tool for Avoiding Polyphenol-Related Enzymatic Inhibition During Mashing

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ABSTRACT

Malted red sorghum (*Sorghum bicolor*) is used as raw material in many traditional beers including Ikigage in Rwanda. In East African countries, sorghum malting may involve adding wood ashes. Mashing traditional sorghum malted with 5% eucalyptus ashes leads to a remarkable increase of EBC congress wort yield (64-69% against 15-22% without ash). Similar mashing yields can be obtained when a ratio of only 20% sorghum with ashes is used, or if ashes are added just prior mashing. Adjusting the wort pH to 6.5 with 1 M NaOH only partially mimics the presence of ashes (mashing yield = 39%). Total polyphenol and total flavanoid measurements, HPLC/UV quantitation of flavan-3-ols and ORAC antioxidant activities enabled us to show that the great benefit of ashes mainly comes from a 63-76% decrease in sorghum polyphenols.

KEYWORDS

Eucalyptus ashes; Ikigage; mashing yield; polyphenols; red sorghum

Introduction

Sorghum (*Sorghum bicolor)* is viewed as one of the major crops contributing to world food security. With production reaching 60 million tons per year, it ranks fifth among the cereals most produced worldwide, after maize, rice, wheat, and barley.[1] In terms of cultivation area, Sudan (7.1 Mha), India (6.2 Mha), and Nigeria (5.5 Mha) are the main producers, while the United States of America ranks first in terms of volume, followed by India and Nigeria.[1]

Sorghum is used in Africa as raw material for various traditional beers. It is the main ingredient for brewing Ikigage, Urwagwa,[2] Inturire, and Inkangaza in Rwanda, Pito and Burkutu in Nigeria and Ghana,[1] Dolo in Burkina Faso,[3] Amgba in Cameroon,[4] Tchakpalo in Benin,[5] Mtama, Indimasi, and Kangala in Tanzania,[6] Impeke in Burundi,[7] Chibuku in Zimbabwe,[8] Merissa in Sudan, and Umqombothi and Kaffir in South Africa.[9]

The use of sorghum in modern breweries still presents many challenges: high starch gelatinization temperature, low amount of β-amylase (prompting the use of exogenous enzymes),[10,11] and high levels of polyphenols, especially in red and brown sorghums, liable to inhibit some mashing enzymes and induce color and colloidal instability in beer.[12] Moreover, combined with high protein level, polyphenols can induce major wort filtration problems, made worse by the absence of husks. On the other hand, sorghum does not contain gluten, so it can be used by people with celiac disease.[13,14] Its high antioxidant activity also motivates its use for producing NABLABs, too often impacted by premature oxidation off-flavors.[15,16]

Sorghum contains a wide variety of polyphenols (Table 1), some of which are rare or absent in other beer ingredients. Sorghum phenolic acids include hydroxybenzoic acids (mainly protocatechuic and p-hydroxybenzoic acid) and hydroxycinnamic acids (mainly ferulic and p-coumaric acid), both free and bound as esters. Sorghum anthocyani(di)ns are unique, as they lack a hydroxyl group at the 3-position of the C-ring. These 3-deoxyanthocyanidins, including luteolinidin and apigeninidin, are more stable than usual anthocyanidins. Flavan-4-ols such as apiforol (leucoapigeninidin) and luteoforol (leucoluteolinidin) are other interesting sorghum polyphenols. Sorghum flavanoids also include, flavan-3-ols ((+)-catechin and (-)-epicatechin)), flavan-4-ones (naringenin and eriodictyol), dihydroflavonols (taxifolin), flavones (apigenin and luteolin), and flavonols (kaempferol).[17] More recently, *trans*-piceid and *trans*-resveratrol were also found in red sorghum grains.[18]

In East African countries, sorghum malting may involve adding wood ashes (especially from eucalyptus) or wood ash extract, either during steeping or just before germination. In Rwanda, the steeped sorghum is mixed with wood ashes on a plastic mat or any other floor cover while spraying germination water.[2] In the malting process for Tanzanian 'Mtama' beer[6] and Impeke from Burundi, sorghum grains are steeped in water already containing wood ashes. According to Nzigamasabo and Nimpagaritse,[19] sorghum should be steeped for 12 h before adding wood ashes, and the steeped grains washed before germination.

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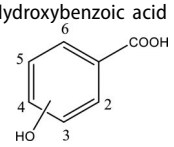
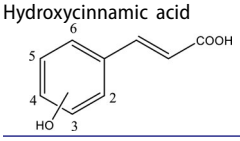
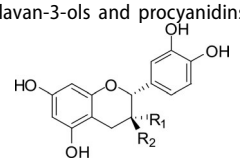
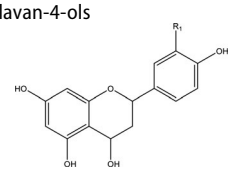
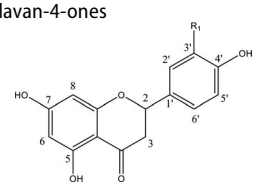
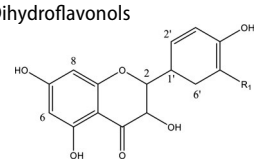
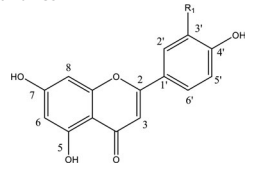
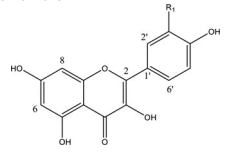
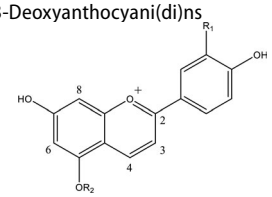
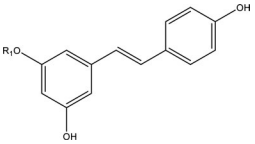
Table 1. Polyphenol chemical structures found in red sorghum grains.

Class and base structure	Compounds
Phenolic acids	
Hydroxybenzoic acid 	Protocatechuic acid (3,4-dihydroxybenzoic acid) 4-Hydroxybenzoic acid
Hydroxycinnamic acid 	p-Coumaric acid (4-hydroxycinnamic acid) Ferulic acid (4-hydroxy-3-methoxycinnamic acid) Sinapic acid (4-hydroxy-3,5-dimethoxycinnamic acid)
Flavonoids	
Flavan-3-ols and procyanidins 	(+)-Catechin R_1=H, R_2=OH (-)-Epicatechin R_2=H, R_1=OH Procyanidin B3 (catechin-α-4,8-catechin) Procyanidin B1 (epicatechin-β-4,8-catechin)
Flavan-4-ols 	Apiforol (leucoapigeninidin) R_1=H Luteoforol (leucoluteolinidin) R_1=OH
Flavan-4-ones 	Naringenin (4',5,7-trihydroxyflavan-4-one) R_1=H Eriodictyol (3',4',5,7-tetrahydroxyflavan-4-one) R_1=OH
Dihydroflavonols 	Taxifolin (2,3-dihydroquercetin) R_1=OH
Flavones 	Apigenin (4',5,7-trihydroxyflavone) R_1=H Luteolin (3',4',5,7-tetrahydroxyflavone) R_1=OH
Flavonols 	Kaempferol R_1=H Quercetin R_1=OH
3-Deoxyanthocyani(di)ns 	Apigeninidin R_1=R_2=H Apigeninidin-5-glucoside R_1=H, R_2=Glc Luteolinidin R_1=OH, R_2=H Luteolinidin-5-glucoside R_1=OH, R_2=Glc
Stilbenes	
	*trans*-Resveratrol R_1=H *trans*-Piceid R_1=Glc

The impact of adding wood ashes or ash extract in sorghum malting is not yet fully understood. According to Irakoze et al.[7] steeping sorghum in wood ash extract (24 h at 30 °C) can reduce tannins by 65%. For others,[20] it may impact enzyme efficiency, mineral composition, or mycotoxin propagation. According to Kyariisima et al., treating sorghum with wood ash extract before germination decreases tannins, thus improving digestibility and metabolization (up to 16.7% for sorghum used in chicken feed formulation).[21]

The aim of this work was to assess the real impact of ashes in brewing applications, added either during sorghum malting or during mashing.

Experimental

Chemicals

Hydrochloric acid 37%, methanol, acetic acid, absolute ethanol 99%, acetone, acetonitrile, dipotassium hydrogen phosphate trihydrate, potassium dihydrogen phosphate, sodium hydroxide, p-dimethylaminocinnamaldehyde, ammonia solution 28–30%, ammonium iron (III) citrate 28%, formic acid, and ethylenediamine tetraacetic acid (EDTA) were purchased from VWR International (Leuven, Belgium). Carboxymethylcellulose sodium salt, 2,2'-azobis (2-methylpropionamidine) dihydrochloride (AAPH), anhydrous magnesium sulfate, fluorescein sodium salt, iron (III) chloride hexahydrate, copper (II) sulfate pentahydrate, calcium chloride dihydrate, polyvinylpolypyrrolidone (PVPP), (+)-catechin, and (−)-epicatechin were purchased from Sigma-Aldrich (Overijse, Belgium). (+)-Taxifolin, and procyanidin B3 were purchased from Extrasynthèse (Rhône, France).

Sorghum and barley malt samples

Unmalted red sorghum samples were collected from four districts in Rwanda: Gicumbi (A and E), Gisagara (B), Kirehe (C), and Rusizi (D). Sorghums were malted according to a slightly modified traditional African sorghum malting process, as described by Lyumugabe et al.[2] For each sample, 1 kg unmalted sorghum sample was sieved and cleaned with tap water, then steeped in 3 L tap water for 24 h at room temperature (20-25 °C). The steeping water was changed after 12 h. No oxygen supply was applied. After steeping, the water was drained out through netted plastic bags for 12 h at room temperature (the sorghum grains had already sprouted by the end of this step). Steeped sorghum grains were spread out on a plastic mat placed on the floor in a dark room (layer thickness around 5 cm), and local eucalyptus ashes were thoroughly mixed with half of them, in the proportion 1:20 (ash weight: dry sorghum weight) while spraying water. All samples were covered with another plastic bag (random small holes to facilitate air exchange) for 72 h (germination stopped when green malt had 1-2 cm radicles). Sorghum (more than 95% germinated) was then dried under the sun (25 °C) for 3 days. Sorghum radicles were removed by rubbing the malted sorghum between the hands and sieving with a netted plastic bag. All malted

sorghum samples were kept at room temperature (20-25 °C) until further use. Barley malt (4°EBC, Pilsen 2RP) was purchased from Castle Malting (Beloeil, Belgium).

Eucalyptus ashes for ICP-MS analysis or addition before mashing

Eucalyptus ashes were provided by a traditional sorghum maltster from Northern province/Rwanda. Ashes were prepared by burning eucalyptus trees (around six years of age) in a cooking stove and sieving to remove unburned and coarse particles. The resulting ashes were kept at room temperature. ICP-MS analyses were operated in a specialized laboratory (LACaMi, UCLouvain, Belgium).

Small scale EBC congress wort production and mashing yield determination

120 g red sorghum malted without eucalyptus ashes (A, B, C, D, and E) or with them (A', B', C', D', and E') was crushed in a disc mill. The EBC congress wort program (45 °C for 30 min, increase to 70 °C at 1 °C/min) was applied to 50 g grist + 200 mL water previously heated to 47 °C. Then 100 mL pure water was added before keeping at 70 °C for 1 h. Liquefaction was checked with iodine after 10 min and then every 5 min. The temperature was gradually reduced to 20 °C and demineralized water was added to reach 450 g. The congress worts were filtered on folded filter papers and kept for determining the pH (with a pH meter), polyphenols, and the mashing yield. The mashing yield (%) was calculated as: (P × (M (g/100g) + 800))/(100-P), with M: moisture content (%, w/w) and P: wort extract (°P).

To investigate the effect of a gradual increase in ash addition on the mashing yield, congress mashing was applied to samples in which the proportion of ash was varied (100% A, 80% *A* + 20% A', 50% *A* + 50% A', 20% *A* + 80% A', and 100% A'). To elucidate whether addition of eucalyptus ashes can have an effect when added during mashing, sample A was mashed without ashes or with 1 or 5% ashes. Similar experiments were conducted by adding 0.1 or 1% PVPP and/or by adjusting the pH to 6.5. Similarly, barley malt was mashed either alone or with 1% PVPP added at unchanged pH or at pH 6.5.

Polyphenol extraction from sorghum malt

Polyphenols were extracted from malted red sorghum according to a modified QuEChERS method (AOAC 2007.01). Briefly, 3 g finely ground red sorghum sample was weighed into a 50-mL Eppendorf conical tube and 10 mL demineralized water was added. The mixture was vortexed for 10 min and ultra-sonicated (Taskforce, ultrasonic cleaner) for 5 min. 10 mL acetonitrile containing acetic acid 1% v/v were added prior to vortexing for 10 min and 5 min in an ultrasonic bath. 5 g anhydrous $MgSO_4$ and 1.25 g sodium acetate were added to the mixture, which was then shaken for 1 min and centrifuged for 5 min at 1500 rpm and 4 °C. The upper extract layer (8 mL) was collected and kept at 4 °C for further analysis (dilution factor: 10/3 from malt to acetonitrile extract).

Polyphenol extraction from EBC worts

EBC congress wort (10 mL) was transferred to a 50-mL Eppendorf conical tube. 10 mL acetonitrile containing acetic acid 1% v/v were added, the mixture was vortexed for 10 min and ultra-sonicated for 5 min. The further steps were as described above for sorghum malt.

Total polyphenol determination

Total polyphenols were quantified according to a slightly modified Bishop assay (EBC method 9.11). Briefly, 1 mL wort, sorghum extract, or blank (distilled water) was mixed with 8 mL CMC/EDTA in a 25-mL volumetric flask. Iron reagent (0.5 mL of a 2% ammonium ferric citrate solution) was added, followed by 9 mL water. The mixture was shaken before adding 0.5 mL diluted ammonia solution (1:3) and distilled water to the volumetric flask mark. After shaking and 10 min rest, the absorbance was measured at 600 nm with a UV/VIS spectrophotometer (UV-3100PC, VWR). Reference solution (Rf) was prepared by omitting the iron reagent. Total Polyphenol Content (TPC in mg/L or mg/kg) was calculated as: (Abs Test – (Abs Rf + Abs Blank)) × 820 × df with df = dilution factor here applied (× 10/3 in case of malt extraction).

Total flavanoid determination

Total flavanoids were analyzed by the p-dimethylamino-cinnamaldehyde method (EBC method 9.12). Briefly, 1 mL polyphenolic extract was transferred into a 10-mL volumetric flask and diluted with distilled water to the mark. 1 mL of this diluted solution (test solution) or 1 mL distilled water (blank) was transferred into a 10-mL test tube and 5 mL chromogenic reagent was added. The mixture was shaken and the solution absorbance was recorded at 640 nm with a UV/VIS spectrophotometer (UV-3100PC, VWR). Total flavanoids (TF in mg/L or mg/kg) were calculated as: (Abs Test – Abs Blank) × 335 × df with df = dilution factor here applied (× 10/3 in case of malt extraction).

ORAC value determination

An automated 96-well plate fluorescence reader (Synergy HT, Bio-Tek, USA) was used. The following solutions in phosphate buffer (75 mM, pH 7.4) were prepared: 55 nM fluorescein (fluorescence probe), 153 µM AAPH (hydroxyl radical generator), various dilutions of Trolox® (8 µM, 16 µM, 24 µM, 32 µM and 40 µM), and suitable dilutions of polyphenolic extract. In each well, 250 µL fluorescein solution and 25 µL sample dilution, blank (phosphate buffer), or standard were added. The plate was then

incubated at 37 °C for 10 min prior to automatic addition of 25 µL AAPH. The fluorescence (480 nm excitation wavelength) was measured immediately and every minute for 50 min. ORAC values (µM Trolox equivalents/g sorghum) were calculated as: $(AUC_{sample} - AUC_{blank})/(AUC_{Trolox} - AUC_{blank}) \times$ Trolox® concentration (µM) × df with AUC = area under the fluorescence curve and df = dilution factor here applied (× 10/3 in the case of malt extraction).

RP-HPLC-UV analysis of flavan-3-ols from sorghum extracts

Prior to HPLC-UV analysis, 200 µL polyphenolic extract was diluted with 300 µL methanol and 500 µL formic acid 0.1% (v/v) and the mixture was filtered on a membrane filter (diameter:15 mm, porosity: 0.45 µm, Macherey-Nagel GmbH & Co., Germany). A 1200 Series system (Agilent, Santa Clara, USA) equipped with a helium degasser, a thermostated G1316A column compartment, a G1329A autosampler, and a G1311A quaternary pump was used. Separation was performed on a C18 Prevail column (150 × 2.1 mm, 3 µm, Hichrom, Berkshire, UK) with a binary eluent system consisting of A: water containing 0.1% formic acid and B: acetonitrile containing 0.1% formic acid. Gradient elution was 97–91% A, 0–5 min, 91–85% A, 5–30 min, 85–67% A, 30–60 min, 67–0% A, 60–62 min, isocratic for 8 min, then return to the initial conditions for 20 min. The column was kept at 25 °C, the flow rate was 0.2 mL/min, and the injection volume was 10 µL. Absorbance spectra were acquired with a G1314C 1260 variable wavelength detector (Agilent, Santa Clara, USA) at 280 nm. The Chemstation software version B.04.02 (Agilent, Santa Maria, USA) was used to control the system and to record data throughout elution. Compound identification was performed by injection of commercial standards. Quantitations in the extracts were carried out according to the calibration curves of (+)-catechin, (−)-epicatechin, procyanidin B3, and taxifolin. Concentrations in malt were obtained by applying a factor of 16.7 (10/3 × 5).

Statistical analyses

All analytical measurements were carried out in duplicate. Multiple comparisons of means were performed with Microsoft Excel (standard deviation determination and *t* test).

Results and discussion

Mashing yield and ash effect

To evidence the effect of ashes on the mashing yield, red sorghum samples collected in four districts of Rwanda, i.e., Gicumbi (A and E), Gisagara (B), Kirehe (C) and Rusizi (D), were malted without (A, B, C, D, E) or with 5% eucalyptus ashes (A', B', C', D', E'). As shown in Figure 1, EBC congress red sorghum worts malted with 5% eucalyptus ashes (A', B', C', D', E') showed substantially higher mashing yields (between 64 and 69%) than worts malted without addition of ashes (A, B, C, D, E) (yields between 15 and

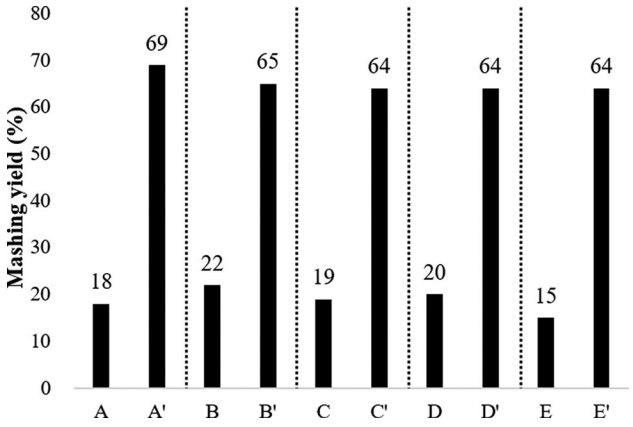


Figure 1. Mashing yield of sorghum malted without (A, B, C, D, E) or with 5% eucalyptus ashes (A', B', C', D', E'). Samples A, B, C, D were taken in September 2023, respectively from the Gicumbi, Gisagara, Kirehe and Rusizi districts in Rwanda. Sample E was taken in Gicumbi in May 2023. Variation coefficient between replicates under 2%.

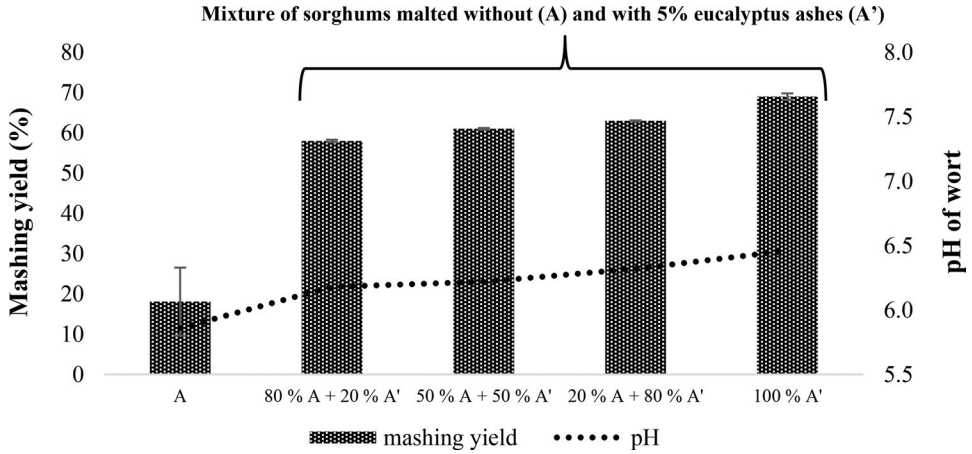


Figure 2. Mashing yields and pH of EBC worts mashed with different proportions of sorghum malted without (A) or with 5% eucalyptus ashes (A'). Variation coefficient between replicates under 2%.

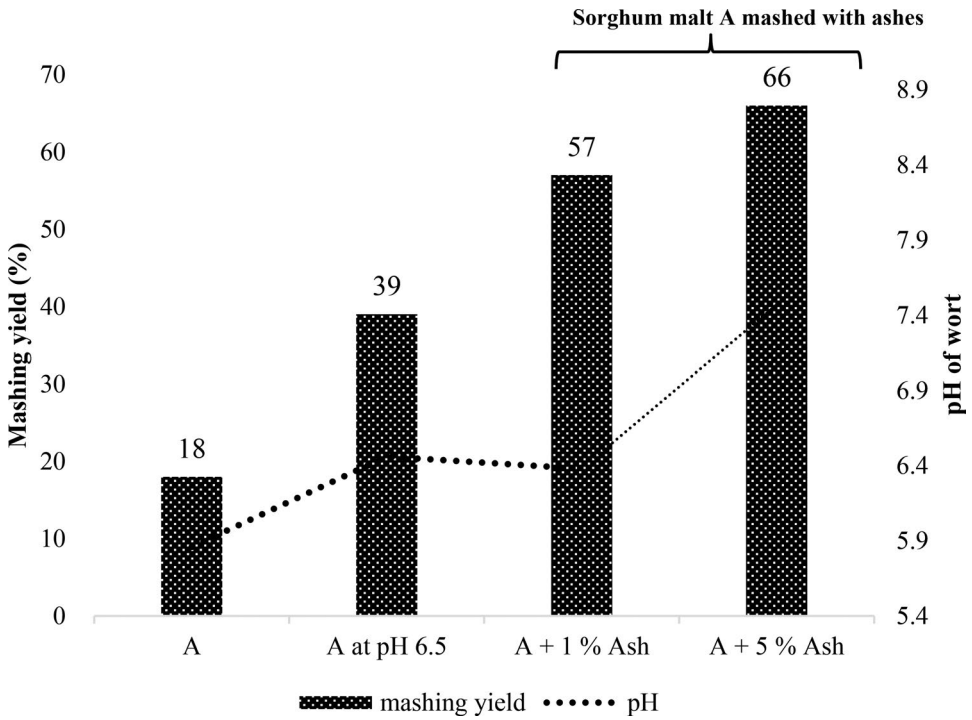


Figure 3. Mashing yield of EBC congress wort mashed with sorghum malt A or with the same malt adjusted to pH 6.5 or spiked with 1 or 5% eucalyptus ashes. Variation coefficient between replicates under 2%.

22%). From these results, it is clear that adding wood ashes during red sorghum malting remarkably increases the mashing yields of EBC congress worts.

Using a mixture of sorghum malted with (A') and sorghum malted without ashes (A) also improved the mashing yield. As depicted in Figure 2, mixing 20% A' with 80% A tripled the mashing yield of congress wort, as compared to mashing sorghum malted without ashes (55% and 18%, respectively).

This led us to suspect that adding ashes during mashing could also improve the yield. This proved to be true: adding 1 or 5% eucalyptus ashes when starting EBC mashing of red sorghum A led to a remarkable increase in yield, from 18% to 57 or 66%, respectively (Figure 3).

Influence of pH on red sorghum mashing

The wort pH was significantly higher in the presence of ashes (which are alkaline[10]), added during malting (Figure 2) or mashing (Figure 3). This prompted us to measure the impact of simply increasing the EBC mashing pH to 6.5 by spiking 1 M NaOH, instead of adding ashes. In this experiment, the mashing yield rose from 18 to 39% (Figure 3). Although this is evidence that the pH is important for enzyme efficiency, the yield reached was below the 66% obtained with sorghum malt A in the presence of 5% ashes.

Effect of eucalyptus ashes on polyphenols

As alkaline pH was not sufficient to explain the strong impact of ashes, total polyphenols and total flavanoids were determined in malted red sorghum (A and A'; Figure 4a) and in the corresponding congress worts (Figure 4b). The results showed that red sorghum malted without ashes (A) exhibited much higher total polyphenol content than red sorghum malted with

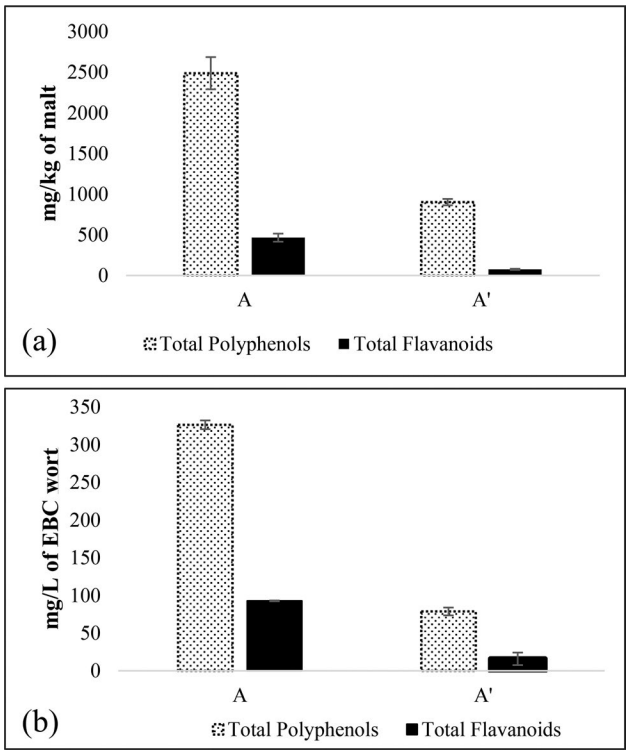


Figure 4. Total polyphenols and total flavonoids (a) in sorghum malted without (A) or with 5% eucalyptus ashes (A') and (b) in congress worts of sorghum malted without (A) or with 5% eucalyptus ashes (A'). Variation coefficient between replicates under 7% for total polyphenols and under 9.5% for total flavanoids.

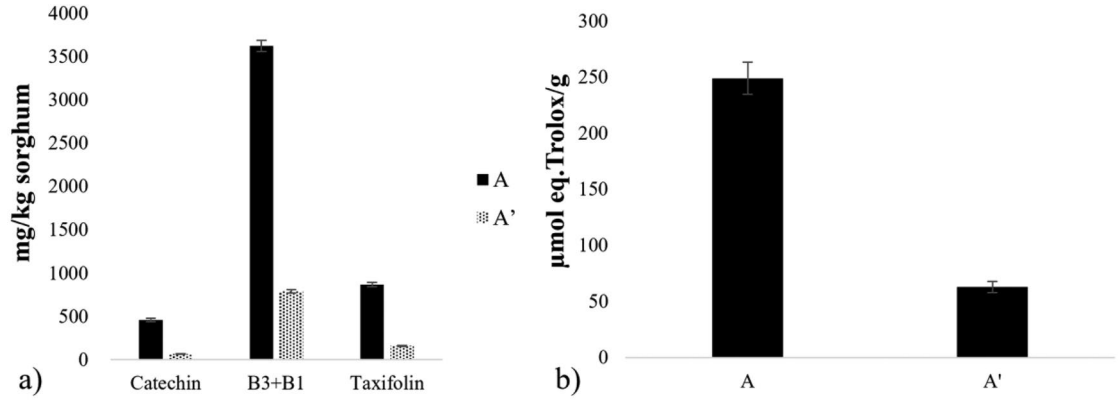


Figure 5. (a) HPLC-UV quantitation of flavan-3-ols and (b) ORAC values of sorghum malted without (A) or with 5% eucalyptus ashes (A'). Variation coefficient between replicates under 5% for HPLC-UV and under 8% for ORAC analysis.

Table 2. ICP-MS mineral content (% w/w) of ashes from eucalyptus harvested in Rwanda.

	Ca	K	Si	Fe	Al	Mg	P	S	Na
% (w/w)	19.3	16.7	9.6	3.7	3.6	2.8	2.5	1.5	0.6
	Ti	Mn	Sr	Ba	Zn	B	Zr	Cr	Cu
% (w/w)	0.3	0.2	0.1	0.06	0.03	0.02	0.02	0.01	0.01

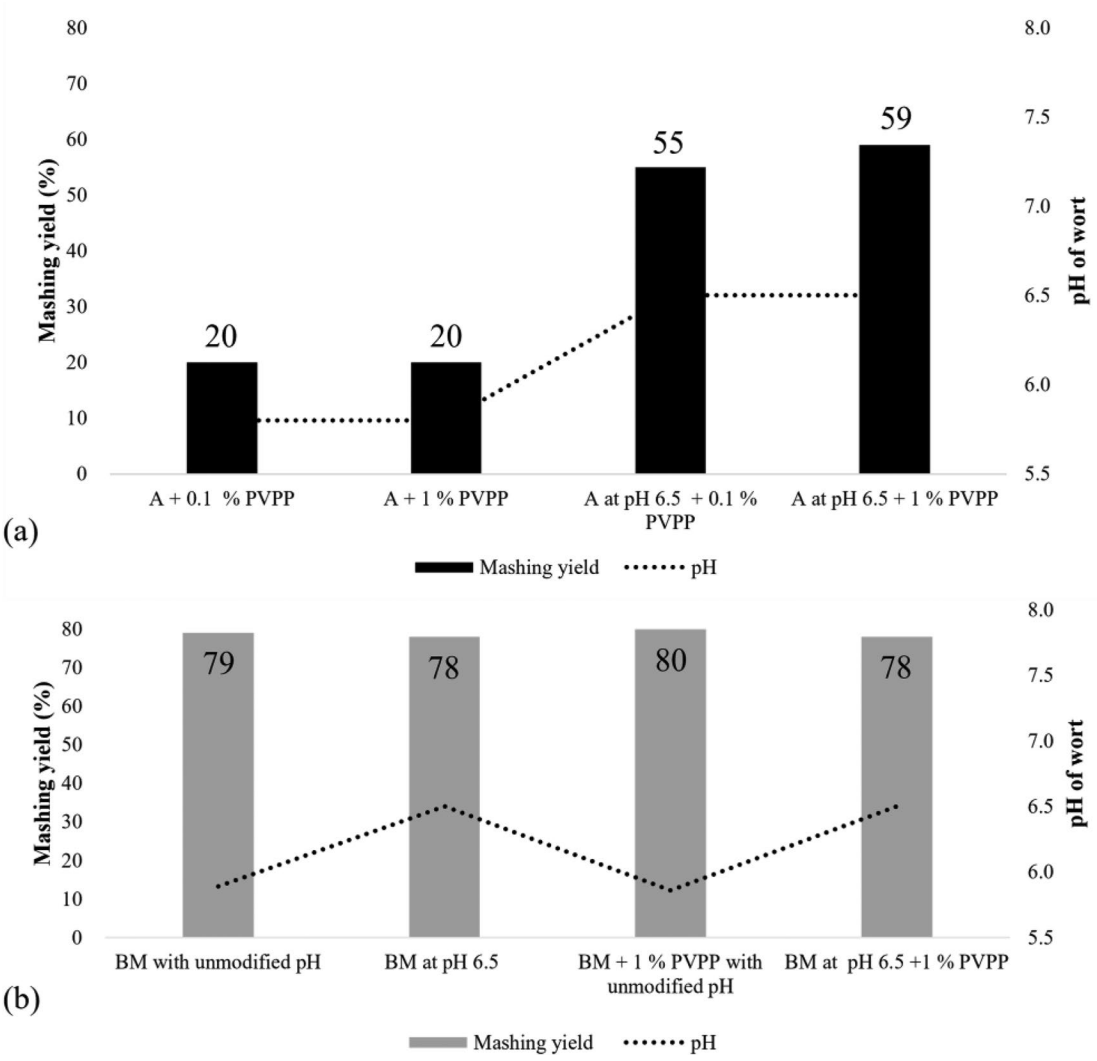


Figure 6. Mashing yields of congress worts mashed with (a) sorghum malt A spiked with PVPP (0.1 or 1% w/w sorghum malt), with or without adjusting the pH to 6.5 with NaOH, or (b) barley malt (BM) spiked or not with PVPP (1 or 5% w/w), NaOH (pH = 6.5), or both. Variation coefficient between replicates under 2%.

ashes (A') (2488 and 902 mg/kg, respectively). Total flavanoids in sorghum malts showed a similar trend, with 465 and 75 mg/kg respectively for A and A'. Congress wort analyses confirmed much lower amounts of total polyphenols (326 and 79 mg/L in A and A', respectively) and total flavanoids (93 and 16 mg/L in A and A', respectively) in the presence of ashes.

The huge loss of total polyphenols (64%) and total flavanoids (84%) was further confirmed by HPLC-UV quantitation of catechin, flavanoid dimers (B1 + B3), and taxifolin. In sorghum A', only 68 mg/kg catechin, 789 mg/kg dimers, and 159 mg/kg taxifolin were found. Logically, the corresponding ORAC antioxidant power also dropped from 249 (for A) to 63 (for A') µmol eq. Trolox/g (Figure 5b).

By increasing pH, ashes could intensify polyphenol enzymatic and chemical oxidations. According to Kim et al.[22] phenolic groups could also bond stably with metal ions present in ashes, especially those having a high charge density (Fe, Cu, Mn, Mo, and Zn), or with metal oxides (TiO_2, Fe_2O_3, Al_2O_3, and MnO_2). Moreover, copper and iron are known as very efficient pro-oxidants.

As depicted in Table 2, the eucalyptus ashes used contained 19.3% Ca (close to the 18.4% published by Pripem et al.[23]) and 17 other elements including K (16.5%), Fe (3.7%), Al (3.6%) and traces of Mn (0.2%), Zn (0.03%), and Cu (0.01%). Just adjusting pH to 6.5 after spiking 96 mg calcium ($CaCl_2.2H_2O$), 18.5 mg iron ($FeCl_3.6H_2O$) and 0.05 mg copper ($CuSO_4.5H_2O$) in the EBC congress mash of sorghum malt A (50 g, without ashes) allowed us to reach a mashing yield of 49%.

We also compared the effect of eucalyptus ashes with that of PVPP, a well-known selective polyphenol binder used for beer stabilization.[24,25] For this, 0.1 or 1% PVPP was added during mashing of A (Figure 6a). The results showed little to no impact on mashing yield (19 and 20%, respectively). On the other hand, mashing sorghum malt A that both contained 0.1 or 1% PVPP and was adjusted to pH 6.5 with 1 M NaOH resulted in mashing yields as high as those obtained for A mixed with 1% eucalyptus ashes (57%). Adjusting the pH thus seems required to allow sorghum polyphenol accessibility and/or oxidation, and further trapping by PVPP.

As depicted in Figure 6b, probably because of much lower polyphenol levels and different enzyme optimal pH values, neither PVPP nor pH 6.5 allowed maximizing the mashing yield of barley malt.

Conclusion

This study investigated the potential use of wood ashes during red sorghum malting and mashing. A remarkable increase in mashing yield was obtained in EBC congress worts in the presence of 1 or 5% eucalyptus ashes, added either during malting or during mashing. In both cases, wort pH increased significantly while polyphenols (quantitated by total polyphenols, total flavanoids, individual flavan-3-ols, and ORAC measurement) strongly decreased. This paper brings, for the first time, scientific data confirming the collective perception among traditional producers of Ikigage that eucalyptus ashes are needed in their recipes. Further research is now needed to determine how, exactly, ashes interact with polyphenols and to what extent they might raise safety concerns.

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