

1 ORIGINAL RESEARCH PAPER

2 **Micronutrient composition and microbial community analysis**
3 **across diverse landraces of the Ethiopian orphan crop enset**

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20 **Abstract**

21 Enset (*Ensete ventricosum*) is a major starch staple and food security crop for 20 million people.
22 Despite substantial diversity in morphology, genetics, agronomy and utilization across its range,
23 nutritional characteristics have only been reported in relatively few landraces. Here, we survey
24 nutritional composition in 22 landraces from three enset growing regions. We present mineral
25 characterization of enset corm tissue, free amino acid characterization of raw and processed
26 (fermented) tissues and genomic analysis of the microbial community associated with fermentation.
27 We show that compared to regionally important tubers and cereals, enset is high in calcium, iron,
28 potassium and zinc and low in sodium. We report changes in free amino acid composition due to
29 processing, and establish that the bacteria genera *Acetobacter*, *Lactobacillus* and *Bifidobacterium*,
30 predominate during fermentation. Nutritional and microbial variation presents opportunities to select
31 for improved composition, quality or safety with potentially significant impacts in food security and
32 public health.

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36 **Keywords**

37 *Ensete ventricosum*; Ethiopia; fermentation; free amino acids; food security; kocho; micronutrients;
38 orphan crops.

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40 **1. Introduction**

41 Humans currently satisfy most dietary requirements with surprisingly few species (Borrell et al.,
42 2019), yet a much greater diversity of nutritionally suitable plants have been identified, often with
43 narrow regions of utilization (Mayes et al., 2012). The food products of the large perennial herb
44 *Ensete ventricosum* (Welw.) Cheesman (Musaceae) are an important dietary starch source in Ethiopia
45 (Borrell et al., 2018; Fanta & Satheesh, 2019; Negash & Niehof, 2004). Commonly known as enset (or
46 alternatively as the false banana or Abyssinian banana), this major food crop is principally cultivated
47 as a highly resilient staple that withstands a wide range of environmental conditions and can buffer
48 seasonal variation in food availability. Enset contributes to the food security of over 20 million people,
49 but is virtually unknown outside of its narrow zone of cultivation in South West Ethiopia, despite
50 growing undomesticated and unutilized across much of East and Southern Africa (Borrell et al., 2018).
51 In addition to being processed for multiple food products, enset is also used for livestock fodder,
52 packaging materials, fiber and traditional medicine (Borrell et al., *in press*; Mohammed, Martin, &
53 Laila, 2013; Olango, Tesfaye, Catellani, & Pè, 2014).

54 Despite the importance of enset for food security and the existence of hundreds of diverse landraces
55 (Borrell et al., 2018), the nutritional composition of the raw plant tissues and processed food products
56 (e.g. *kocho*) has only been reported in a small number of landraces (Bosha et al., 2016; Daba &
57 Shigeta, 2016; Mohammed et al., 2013; Nurfeta, Tolera, Eik, & Sundstøl, 2008). Reported nutritional
58 composition and the relative concentrations of certain micronutrients show considerable variation.
59 Whilst this may be attributable in part to differing analysis methods, the extensive diversity of
60 genetically differentiated enset landraces (Borrell et al., 2018; Tobiaw & Bekele, 2011), heterogeneity
61 of farm management practices (Garedew, Ayiza, Haile, & Kasaye, 2017; Olango et al., 2014) and

62 environmental conditions, particularly soil (Amede & Diro, 2005; Borrell et al., 2018), may also be a
63 contributing factors. Therefore, we highlight the need to profile micronutrient composition across a
64 representative subset of enset landrace diversity, whilst also characterizing the ubiquitous effects of
65 fermentation on the composition, relevant to the quality and safety of enset foods, and the
66 associated microorganism diversity responsible for mediating tissue processing.

67 The edible parts of enset comprise the starch rich pseudopetioles forming the pseudostem
68 (overlapping leaf sheaths) which are decorticated, and the corm (the underground base of the stem
69 that serves as a storage organ) which is pulverized and pressed (Borrell et al., 2018). These two main
70 tissues are collectively processed, using fermentation pits, into starch staples including *bullā* and
71 *kocho* (see Birmeta, Bakeeva, & Passoth, 2018, for a detailed description). *Kocho* is the bulk of the
72 fermented product and is baked into a thin fibrous bread considered to have a good shelf life. *Bullā* is
73 a small amount of water-insoluble starchy product separated from the *kocho* during processing by
74 squeezing and sometimes consumed separately. The corm of enset is also occasionally consumed
75 boiled, much like potato, and this is called *amicho*.

76 The precise fermentation practice is variable among regions and cultural groups (Garedew et al.,
77 2017; Hunduma & Ashenafi, 2011). Karssa et al., (2014) reports preparation of a starter culture
78 (known as *gamancho* or *gamma*) from selected corms of mature plants, followed by a two-phase
79 process, with surface and then pit fermentation. Bosha et al. (2016) reports a ground mix of several
80 other plant species being added to the mashed corm to initiate fermentation. Whereas many other
81 authors (Birmeta et al., 2018; Gashe, 1987), indicate that fermentation is initiated simply from
82 mashed tissue left for several days at ambient temperature.

83 The microorganisms responsible for fermentation alter the chemical composition of the raw
84 substrate, which in some cases enriches the nutritional value of fermented products (Tamang,
85 Watanabe, & Holzapfel, 2016) by removing anti-nutritionals and breaking down complex components.
86 Furthermore, microbial communities introduced during processing are often critical to food safety
87 and preservation by preventing growth of spoilage and toxic organisms. These microorganisms, often
88 occurring as communities in food products are poorly known in orphan and minor tropical crops
89 cultivated by subsistence farmers (Tamang et al., 2016). However, improvement of these cultures
90 represents a relatively accessible opportunity to enhance the nutritional consistency, bioavailability
91 and quality of neglected food products (Chelule, Mokoena, & Gqaleni, 2010), while urbanization and
92 penetration of shops leads to loss of cultures traditionally maintained by smallholders.

93 In a study on *kocho* production from enset, Gashe (1987) reported that *Leuconostoc mesenteroides*
94 and *Streptococcus faecalis* are responsible for initiating fermentation and reducing the pH. These are
95 then superseded by the homofermentative bacteria *Lactobacillus coryniformis* subsp. *coryniformis*
96 and *L. plantarum* which reduced the pH further. More recently, in an analysis of kocho samples from
97 the Wolkite area, Weldemichael, Shimelis, Emire, & Alemu (2019) identified kocho-associated
98 bacteria as predominantly from the genera *Lactobacillus* and *Acetobacteria*, whilst a survey of kocho
99 samples from the Gamo highlands identified high relative abundance of *Enterobacteriaceae* and *L.*
100 *mesenteroides* subsp. *cremoris* in the early stages of fermentation (Andeta, Vandeweyer,
101 Woldesenbet, Eshetu, & Hailemichael, 2018). More generally, the process of fermenting enset has
102 been reported to reduce total protein and carbohydrates, whilst increasing free amino acids (FAAs)
103 1.6-fold (Urga et al. 1997). However, the specific free amino acids in fermented products have not
104 been characterized. Therefore whether enset is a source of essential amino acids (those the body
105 cannot synthesize in sufficient quantities) in this form is unknown.

106 We hypothesize that significant genomic, phenotypic, environmental and agronomic variation in
107 enset should also result in variation in nutritional composition across landraces. In this study we
108 investigate selected micronutrients with a focus on inorganic heavy metal and trace elements and
109 free amino acids in domesticated enset in Ethiopia. We profile both raw tissues and processed
110 (fermented) products in samples from 22 landraces across three major enset growing regions, and
111 present a quantitative genomic survey of the microbial community associated with enset
112 fermentation. We place our results in the context of other regionally available staples and the
113 opportunities that nutritional and microbial variation presents for applied biotechnology to improve
114 the nutritional quality, consistency and safety of enset derived foods.

115 **2. Material and Methods**

116 **2.1 Sample collection and preparation**

117 Fresh samples of six enset tissues (corm, pseudostem, leaf, fruit flesh, fruit peel (exocarp) and seed)
118 were harvested from a selection mature enset plants (n=28, landraces=22) occurring in three regions;
119 Sidama, Wolaita and Gurage zones in the Southern Nations Nationalities and Peoples Region (SNNPR),
120 Ethiopia (Table 1). In selecting samples we sought to capture the full range of phenotypic and cultural
121 variation. Selected plants were individually prepared, processed and fermented following traditional
122 local practices and associated qualitative indigenous knowledge related to processing was recorded.
123 Subsequently, samples of three enset products *kocho*, *bulla* and mixed *kocho* and *bulla* (Ko-Bu;
124 whereby the *bulla* liquid is not isolated during preparation, a common practice in parts of Sidama)
125 from selected plants were collected post-fermentation, air dried and powdered using an electric
126 grinder.

127 **2.2 Micronutrient and mineral composition**

128 Micronutrient content used only raw corm tissue, as relative concentration of micronutrients should
129 not be affected by fermentation. Total ash content was estimated using 5 g of desiccated corm tissue.
130 Samples were charred in a hot plate (Wagtech Model ST15, Sweden) and ignited at 550°C for 5 hours
131 a in a Muffle furnace (Carbolite Model S302RR, Sweden). Residual ash was calculated as a percentage
132 of the original desiccated sample weight.

133 Mineral content was assessed using 0.5 g corm samples digested in a closed high-performance
134 microwave digestion system (ETHOS) with the addition of trace metal grade 65% HNO₃ (Fisher
135 Scientific, UK) and H₂O₂ according to the manufacturer's recommendations under high pressure and
136 temperature. The digest was washed with distilled water (Milli-Q). Multi-elemental analysis was
137 conducted using an inductively coupled ICP-MS (Thermo-Fisher Scientific iCAP-Q; Thermo Fisher
138 Scientific, Bremen, Germany). Samples were introduced from an auto-sampler (Cetac ASX-520)
139 incorporating an ASXpress™ rapid uptake module through a perfluoroalkoxy (PFA) Microflow PFA-ST
140 nebuliser (Thermo Fisher Scientific, Bremen, Germany). Internal standards were introduced to the
141 sample stream on a separate line via the ASXpress unit and included Ge (10 µg L⁻¹), Rh (10 µg L⁻¹) and
142 Ir (5 µg L⁻¹) in 2% trace analysis grade (Fisher Scientific, UK) HNO₃. External multi-element calibration
143 standards (Claritas-PPT grade CLMS-2 from SPEX Certiprep Inc., Metuchen, NJ, USA) including Al, As,
144 Cd, Ca, Co, Cr, Cs, Cu, Fe, K, Mg, Mn, Mo, Na, Ni, P, Pb, S, Se, and Zn, in the range 0 – 100 µg L⁻¹ (0, 20,
145 40, 100 µg L⁻¹) were used. A bespoke external multi-element calibration solution (PlasmaCAL, SCP
146 Science, France) was used to create Ca, Mg, Na and K standards in the range 0-30 mg L⁻¹. In addition,
147 KH₂PO₄ and K₂SO₄ solutions were used to calibrate the machine during determination of P and S,
148 respectively. Sample processing was undertaken using Qtegra™ software (Thermo-Fisher Scientific)
149 utilizing external cross-calibration between pulse-counting and analogue detector modes when
150 required. Blank digests containing all except enset samples were used for quality control. In addition,

151 a certified reference material (SRM 1567b – wheat flour) from the National Institute of Standards and
152 Technology (NIST), USA was used for standardization. Comparative plant nutritional composition data
153 was sourced from the USDA Food Composition Databases (USDA, 2012) and Feedipedia (Heuze,
154 Thiollet H., Tran G., Hassoun P., & Lebas F., 2017), and plotted together with data from this study.

155 **2.3 Crude protein and free amino acid composition**

156 Crude protein was estimated using 0.5 g of desiccated corm tissue. Briefly, 6ml of conc H₂SO₄ and
157 3.5ml of H₂O₂ were added followed by 3 g of a catalytic mixture of CuSO₄ and K₂SO₄ (1:15) for 15
158 minutes. The solution was heated at 370°C using a kjeldahl digester until a clear solution was
159 observed and distilled using the Kjeldahl distillation apparatus (Auto K9840 Analyzer, Kjeltec, BCR-
160 Technology Co, Ltd., China). The auto distiller was adjusted to add 30 ml distilled water (to avoid
161 precipitation of sulfate in the solution), and 40 ml NaOH (35%) in order to neutralize excess acid,
162 break down ammonium sulfate and release ammonia gas. The distillate was collected for 8 minutes in
163 a 250 ml Erlenmeyer flask containing 2% boric acid and five drops of methyl red indicator. The
164 distillate was titrated with standardized 0.1M HCl and sample N content determined by subtracting
165 the N content of blank control samples.

166 Free amino acid composition was assessed in a subset of samples using an EZ:faastTM kit
167 (Phenomenex, Macclesfield, UK). Powdered samples (100 mg) were extracted in 100% water and
168 sonicated for 20 min prior to centrifugation. Supernatants were derivatized using the method
169 described in the manufacturer's instructions, and as described previously (Dziągwa-becker, Weber,
170 Zajączkowska, & Oleszek, 2018). Analyses of derivatized amino acids were performed with 2µl sample
171 injections on a Thermo Scientific system, consisting of an 'Ultimate 3000' UHPLC unit and an 'LTQ
172 Velos Pro' mass spectrometer (Thermo Scientific, Waltham, MA, USA). Chromatography (qualitative

173 and quantitative analyses) was performed as described in the EZ:faast™ kit's instructions, with amino
174 acids assigned by comparison with the calibration amino acid standards provided with the kit. For
175 each enset sample, all analyses were performed in triplicate, and the base peak areas for detected
176 amino acid derivatives analyzed using Thermo Xcalibur (Thermo Fisher Scientific, USA) and R software
177 (R Core Team, 2017), and averaged per sample.

178 **2.4 Genomic characterization of enset fermentation**

179 DNA was extracted from kocho using a standard CTAB protocol. High quality DNA was successfully
180 isolated from the kocho of three landraces (Ganticho, Agade, and Ado), one sample failed (Hala).
181 Library preparation followed the double digest restriction site associated sequencing protocol (tGBS)
182 of Data2Bio (Iowa, USA). Sequencing was performed using the Ion Proton platform (Thermo Fisher
183 Scientific, USA). Raw reads were subsequently trimmed and quality controlled following the Data2Bio
184 pipeline.

185 To identify microorganisms present in kocho samples, retained sequences were mapped to 24,610
186 reference bacteria and 285 reference fungi genomes, obtained from NCBI
187 (<https://www.ncbi.nlm.nih.gov/>, accessed 20th March 2019). A local blast database was built and
188 blast searches performed with e-value threshold of e^{-20} and scoring (match-mismatch) =1-2. The
189 percentage of retained reads that aligned to a bacterial or fungal genome were recorded. As a
190 control, DNA was also extracted from the leaf tissue of multiple farmer-grown enset accessions and a
191 similar pipeline used to characterize bacteria or fungi present on the surface or within plant tissue.
192 This approach provides a comparative line of evidence that where specific microbes identified in
193 kocho samples are present at high concentrations, this is indicative that they are associated with
194 fermentation (rather than simply present). It also provides an indication on whether the microbial

195 community responsible for fermentation originates from the plant surface/tissue or elsewhere, such
196 as the wider environment (e.g. soil) or a starter culture.

197 **3. Results and discussion**

198 **3.1 Sample collection, processing and associated knowledge**

199 Fermentation was initiated from mashed pseudostem tissue left for several days at ambient
200 temperature without contrived additives of cultures or previous products. Kocho and Bulla were then
201 fermented for three months, and Ko-Bu was fermented for six months. We recorded the ubiquitous
202 use of fresh enset leaves as a work surface for processing and the prevailing views that fermentation
203 will not be successful if the fermentation pit is not positioned within the enset growing area.
204 Positioning of the enset fermentation pit within the enset growing area may indicate that
205 temperature (or shade) is important, or alternatively may be related to the presence of specific
206 microorganisms in the soil or elsewhere in the environment associated with enset plants. The
207 resulting fermentation products can be stored for long periods (>6 months). As expected, farmers
208 reported that different enset landraces produce *kocho* (or other products) of different qualities or
209 preferred uses (including as medicines), however multiple landraces are normally processed together.
210 In times of extreme famine, wild enset may be blended with harvested domestic plants, despite
211 generally being considered unpalatable, though the resulting product is regarded as having lower
212 quality, perhaps due to taste.

213 **3.2 Inorganic composition of enset and comparison with other regional crops**

214 Mean ash content across 19 corm samples was 5.0% (sd=1.4%), which is similar to previously
215 published ash content (4.5%) for enset corms (Nurfeta et al., 2008). Concentrations of inorganic

216 micronutrients and trace elements in raw enset corm tissue were averaged across 19 corm samples,
217 each with two replicates, and plotted in Figure 1A. Inorganic micronutrient composition varied both
218 by individual sample, and region (Figure 1B), although these data are unable to distinguish the relative
219 importance of these two factors. Samples originating from Gurage zone were most variable on the
220 first axis (27.1% of the variation), whilst samples from Sidama vary on the second axis (22.7% of the
221 variation).

222 These data illustrate important variation in micronutrient composition across enset landraces and
223 growing regions, which mirrors the high vernacular and genetic diversity reported elsewhere for this
224 crop (Borrell et al., 2018; Yemataw, Mohamed, Diro, Addis, & Blomme, 2014). This indicates the
225 influence of genetic, environmental and/or management factors, and as a result, there are likely to be
226 appropriate genetic targets for breeding to enhance nutritional composition. Similar performance
227 gains have been achieved in a range of other species for which there is high variability in
228 micronutrient composition (Welch & Graham, 2004). These data also suggest that climate or soil
229 conditions, together with enset management practices may influence the uptake and accumulation of
230 micronutrients in enset corm tissue, in particular because micronutrient composition appears to
231 cluster weakly by region (Figure 2) – though many landraces are region specific making further
232 investigation of this pattern challenging. We note that consumption of some micronutrients in high
233 amounts is not recommended (e.g. Ni, Cd) and high levels of these metals in samples from Sidama
234 (Figure 1B, 1C) may be associated with groundwater contamination.

235 In a comparison with regionally important tuber and cereal crops, enset reports particularly high
236 values for calcium, iron, potassium and zinc and relatively low values for sodium (Figure 2). Both iron
237 and zinc deficiencies are widespread in the region (Grebmer et al., 2018). Anemia (iron deficiency) is

238 reported in 56% of children and 24% of adult women in Ethiopia (Gebru et al., 2018), with anemia also
239 included in the World Health Organization's Global Nutrition Monitoring Framework for Ethiopia
240 (WHO, 2019). This emphasizes the importance of enset as a potential dietary source of iron. Iron
241 concentrations have previously been reported as higher in enset pseudostem compared to corm
242 (Heuze et al., 2017) and we find further support for this pattern across a larger range of landraces
243 (Figure 2), though we show almost 8-fold variation between samples. Zinc deficiency is also reported
244 in diets in Ethiopia (Gebru et al., 2018; Kebede & Modes, 2013). In this study we provide further
245 evidence that enset is an important source of Zinc, and that enset corm contains higher levels of zinc
246 than pseudostem tissue. Overall, most micronutrients occur at higher concentrations in the
247 pseudostem than the corm (Figure 2).

248 **3.3 Organic composition and the nutritional implications of enset fermentation**

249 Mean total protein content across 19 corm samples in this study was 4.6% (sd=1.5%), broadly
250 consistent with values published elsewhere (Feedipedia: 3.5%, sd=1.1%). The mean concentration of
251 FAAs across raw enset tissues and processed food products are reported in Figure 3A with overall
252 concentrations for each FAA shown in Figure 3B. Of the edible components of enset, corm tissue
253 contained the highest concentrations of FAAs, but was also most variable. FAA variation across
254 samples is presented in Figure 3C with variable loadings for the first and second principal components
255 reported in Figure 3D. Based on principal component analysis, concentrations for pseudostem were
256 mostly clustered but showed higher variation for corm tissue. The most variable FAAs on the second
257 axis associated with corm variability included arginine, histidine and aspartic acid. The relative
258 concentrations of individual FAAs before and after fermentation is reported in Figure 4.

259 Processing of crops is normally performed to improve the digestibility of the product, removing anti-
260 nutritionals and allowing storage without microbial or animal (including insect) contamination.
261 However, there is no evidence that unprocessed enset is toxic, contrasting with cassava (cyanide) or
262 many legumes (with both highly toxic lectin glycoproteins and frequent association with fungi
263 producing mycotoxins). In this instance, enset processing appears to have a significant impact on the
264 composition of derived food products, with FAAs present at higher concentrations in raw tissues
265 (particularly leaf and corm tissue; Figure 4A), suggesting that microorganisms are net consumers of
266 FAAs during enset fermentation. These data also show that certain FAAs may be localized to specific
267 tissues, for example arginine and glutamine are both detected in corm, but at low concentrations or
268 absent in other tissues both pre- and post-fermentation (Figure 4). Similarly, phenylalanine and
269 glycine occur at higher concentrations in fermented products than raw tissues, whilst many other
270 FAAs decline. Overall, several essential amino acids (isoleucine, leucine, phenylalanine and valine) are
271 increased in fermented products, particularly the main staple food, *Kocho*, suggesting that traditional
272 fermentation practices may contribute to nutrition.

273 When protein or amino acids are ingested, the vast majority of digestion products that reach the
274 blood stream are single amino acids, with the completeness of protein digestion dependent on
275 metabolic factors (Bhutta & Sadiq, 2012). Thus in some cases ingested FAAs may be more bioavailable
276 whilst in others a protein-rich diet may be poorly digested and of reduced nutritional value (Bhutta &
277 Sadiq, 2012). Furthermore, certain amino acids contribute to the flavour of foods (Kato, Rhue, &
278 Nishimura, 1989), thus influencing the selection of processing or fermentation methods and dietary
279 choices of consumers, with potential consequences for human nutrition. We note wild enset is not
280 consumed because it is considered bitter and unpalatable. It is interesting, therefore, that
281 fermentation appears to markedly reduce aspartic acid and glutamic acid, both of which produce a

282 potent sour taste at relatively low concentrations, as well as reducing arginine and histidine
283 concentrations, which are characteristic of bitter tastes. Whether development of processing
284 techniques has been concomitant with domestication to produce food with improved palatability is
285 an interesting area for further research.

286 This study also reveals detection of varying concentrations of the essential amino acids isoleucine,
287 leucine, lysine, phenylalanine, threonine, tryptophan, and valine in different enset tissues (Figure 4),
288 in the form of FAAs. Consequently, these data provide the first evidence that enset, as part of a
289 broader diet, may contribute to intake of these essential amino acids. The principal FAA detected
290 across all enset samples analysed in this study was arginine (Figure 4), which occurred at the highest
291 levels in corm tissue. Although arginine is not an essential amino acid, some evidence suggests that
292 increased dietary arginine can improve outcomes in critically ill individuals (Emery, 2012) and it is
293 considered essential for infant growth, with histidine also being important for the latter (Brayfield,
294 2019), and also detected in enset tissue, especially the corm (Figure 5).

295 A traditional use of enset in Ethiopia is its intake in the form of amicho (boiled corm), which is reputed
296 to heal bone fractures (Borrell et al., 2018). Arginine is involved in collagen formation, tissue repair
297 and wound healing via proline, which is hydroxylated to form hydroxyproline, and it may also
298 stimulate collagen synthesis as a precursor of nitric oxide (Van de Poll, Luiking, Dejong, & Soeters,
299 2012). The analysis of free amino acids in this study has revealed high levels of arginine, compared to
300 other amino acids, detected in different edible parts of enset. This novel finding provides a rational
301 scientific basis for the first time that may explain the reputed traditional use of enset to aid bone
302 healing after breakage. Intriguingly, three landraces often reported as having medicinal properties
303 (Koshkowashiye (Gurage), Astara (Sidama) and Lochingiya (Wolaita)) report three of the four highest

304 arginine values, whilst Lochingiya and Astara also report the highest calcium concentrations, a mineral
305 critical for bone development if not healing.

306 **3.4 Microbial community characterization and signatures of contamination**

307 Sequencing of kocho samples resulted in 9.6M raw reads. After quality control 8.6M reads were
308 retained with a length of 50-209bp. Of these, 1.9M and 24,300 reads returned BLAST hits above a
309 threshold of e^{-20} for bacteria and fungi genomes, respectively. The bacterial species *Acetobacter*
310 *pasteurianus* was the most frequent in the kocho samples Ganticho and Ado, whereas *Raoultella*
311 *ornithinolytica* was found to be most frequent in Agade (Figure 5A). The fungal species *Penicillium*
312 *chrysogenum*, *Pichia kudriavzevii* and *Aspergillus fischeri* were found most frequent in the Ganticho,
313 Ado and Agade samples respectively (Figure 5B). A total of 797 bacterial species were found in
314 common across the three kocho samples, though comparatively only eight fungi species were found
315 across all samples (Figure 5C). As a control, BLAST results from leaf tissue is provided in Figure 5D and
316 largely identifies distinct species from the main kocho analysis. The same analysis is presented at
317 genus level in Figure S1.

318 Genomic analysis of the microbial community associated with enset fermentation showed that the
319 most abundant genus of bacteria was *Acetobacter*, which is generally aerobic and known for
320 producing acetic acid, reducing the pH of the fermenting enset pulp. It both gives a desirable flavor to
321 the product and lowers the pH which inhibits growth of other organisms, thus allowing safe storage of
322 the product. Whilst these species may occur as endophytes, the most common species identified in
323 the kocho samples (*Acetobacter pasteurianus*) was not identified in raw leaf tissue (Figure 5D).
324 The second most abundant genus was *Lactobacillus*, a group of anaerobic bacteria often associated
325 with controlled fermentation in foods. In a comparison with the bacteria identified by Gashe (1987)

326 we also found *L. mensenteroides*, *L. coryniformis* and *L. plantarum* but not *S. faecalis*. In the genome
327 sequencing of leaf-derived DNA, Harrison et al., (2014) reported extensive hits (>8%) against
328 *Pseudomonas fluorescens* and *Methylobacterium radiotolerans*, which they propose are endophytes
329 associated with enset; we also find a small number of *P. fluorescens* hits in our kocho samples, but
330 only one hit to *M. radiotolerans*. Overall, our analysis identified many more bacterial than fungal
331 sequences. We also find a much higher proportion of bacteria species in common between samples
332 (39.2%), compared to fungi (1.6%) including the yeasts. This suggests that bacteria are principally
333 responsible for enset fermentation.

334 These analyses provide an opportunity to identify potentially harmful microorganisms such as those
335 associated with spoilage or food poisoning (Bhunja, 2018). The number of hits associated with
336 *Escherichia*, *Campylobacter*, *Salmonella*, *Clostridium* (except in the *Ganticho* kocho sample) and
337 *Listeria* was generally very low in all samples. However, whilst the microbial community composition
338 of *Ganticho* and *Ado* were very similar, *Agade* was dominated by *Raoultella ornithinolytica*, a species
339 associated with human infections. *Stenotrophomonas maltophilia*, *Acinetobacter johnsonii*, *Rahnella*
340 *aquatilis* are other species potentially harmful to health were also among the most frequently
341 identified bacteria in this sample. Similarly, the highest fungal hit for *Agade* was *Aspergillus*
342 (*Neosartorya*) *fischeri*, a close relative of the major pathogen *Aspergillus fumigatus* also associated
343 with hypersensitivity pneumonitis (farmer's lung, immunologically mediated inflammation). This
344 suggests that in comparison to *Ganticho* and *Ado*, *Agade* could be characterized as a contaminated
345 food product. The prevalence of enset food product contamination in Ethiopia is unknown.

346 **4. Conclusions**

347 Ethiopia has historically been the world's largest recipient of targeted food aid (World Food
348 Programme, 2013) and is 93rd of 119 qualifying countries in the 2018 Global Hunger Index (Grebmer
349 et al., 2018). Nationally, undernourishment affects 21.4% of the population and 38.4% of children
350 under the age of five are affected by stunting (Grebmer et al., 2018). Therefore effective utilization of
351 agricultural diversity is a priority to achieve food security and address public health needs, particularly
352 in the context of climate change and population growth (Pironon et al., 2019). However despite being
353 a center of diversity for plant domestication, dietary diversity over much of Ethiopia is extremely low
354 due to overdependence on starchy staples (Gebru et al., 2018) with major dietary deficiencies in iron
355 and zinc. This is indicative of a However, whilst child stunting prevalence in the enset growing region
356 (reflecting *chronic* undernutrition) is largely consistent with the national average (CSA and ICF, 2016),
357 these areas have some of the lowest national levels of child wasting (an indicator of *acute*
358 undernutrition). This provides compelling indirect evidence of the food security potential of enset in
359 mitigating acute food insecurity events.

360 The results presented here show that there is significant potential for enhanced nutritional benefits
361 (e.g. iron, zinc, FAAs) from enset that could impact the chronic health and welfare challenges
362 experienced by millions of Ethiopian farmers whom rely on enset as a resilient starch staple. We show
363 that there is significant variation in enset nutritional diversity, partitioned across multiple stages of
364 enset cultivation and processing from the selection of landraces, environmental conditions and
365 management practices, to the timing and selection of tissues for harvest and the microbial
366 community associated with enset processing. Many of these sources of variation are not currently
367 understood, controlled or investigated and represent significant opportunities for optimization or
368 improvement. We also highlight that in addition to there being few enset germplasm collections
369 (Borrell et al., 2018), there are no collections of the microbial communities associated with kocho

370 processing. We therefore suggest that it is important to further document, collect and preserve their
371 diversity as they would be lost should farm structures, agronomy or management change for social or
372 economic reasons. In summary, more than 20 million Ethiopians rely on enset-derived products as a
373 starch staple or co-staple, and this population is projected to grow significantly in the coming decades
374 (Borrell et al., 2018). Therefore, selection of enset landraces with improved raw nutritional content or
375 enhanced processing techniques that improve the composition, quality or safety of enset based foods
376 has the potential for significant public health impacts.

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391 **Ethics Statement**

392 All indigenous knowledge associated with enset was collected with prior informed consent and in
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398 **Conflict of Interest Statement**

399 The authors declare no conflict of interests.

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549 Tables

550 **Table 1.** Enset tissue and food product types evaluated in this study.

Type	Tissue	Samples	Uses and food products
Tissue	Corm	19	A raw, underground storage organ used to make both kocho and bulla. Also boiled and eaten as 'Amicho'.
	Pseudostem	9	A raw aboveground tissue comprising overlapping leaf sheaths. The pseudostem is the main tissue type making up the bulk of both kocho and bulla. Petioles and leaf midribs are fed to cattle as fodder.
	Leaf	1	Used as a protective layer in baking bread and to line the kocho processing area and fermentation pits
	Fruit flesh	1	Not currently utilized
	Fruit peel	1	Not currently utilized
	Seed	2	Not currently utilized
Product	Kocho	11	A traditional fermented flatbread
	Kocho-Bulla (KoBu)	8	A traditional fermented flatbread, but made without extracting the bulla liquid. In this study this practice occurs in Sidama region
	Bulla	8	The water-insoluble starchy liquid extracted from kocho by squeezing. Bulla can be dried and used as a flour, or made into a gelatinous porridge. It is produced in small amounts and often considered the most valuable enset food product.

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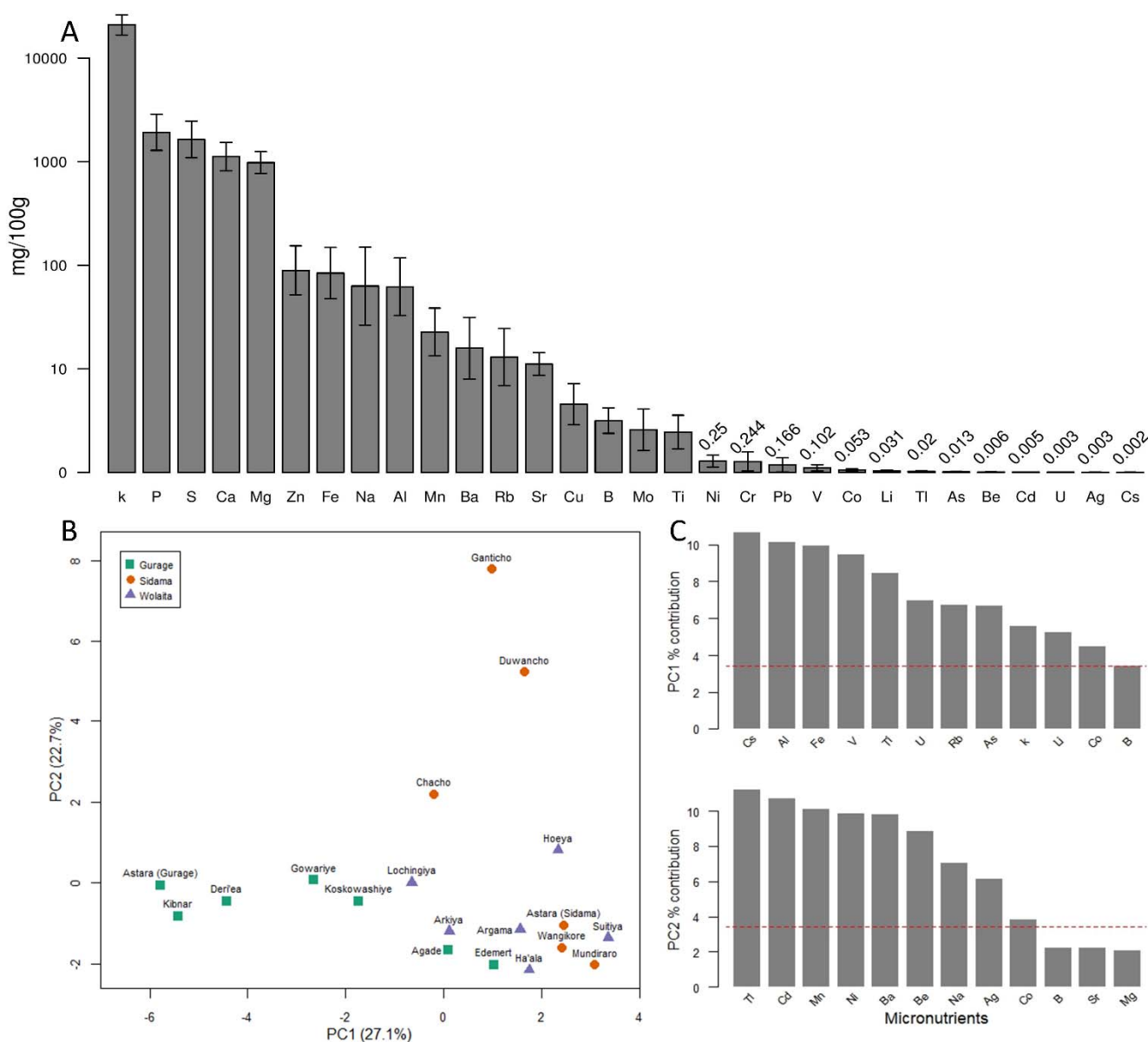
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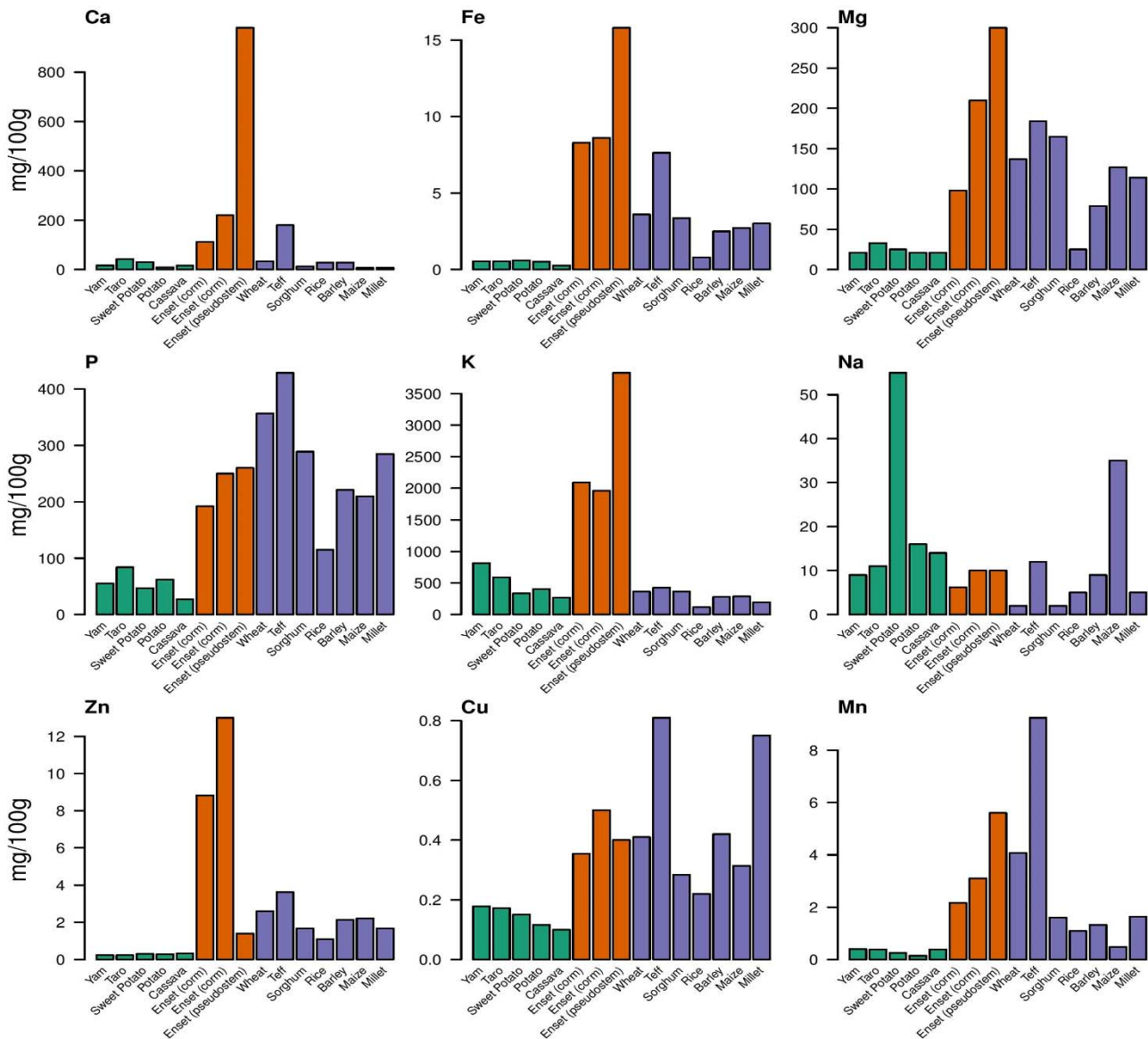
558 **Figures**



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560 **Figure 1. Concentration of inorganic minerals in enset corm tissue.** A) Mineral values averaged
 561 across 19 enset corm samples. Bars denote standard deviation. B) Principal component analysis of
 562 enset corm inorganic composition across three geographical regions. C) Axis loading plots for the first
 563 and second principal components. The horizontal red line shows the level at which axis contributions
 564 would be equal.

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Figure 2. Comparison of enset tissue inorganic micronutrients (corn and pseudostem) with

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regionally occurring tubers and cereal crops. For enset three values are provided (from left to right);

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i) Enset (corn) results from this study (note: unavailable for K), ii) Enset (corn) from published

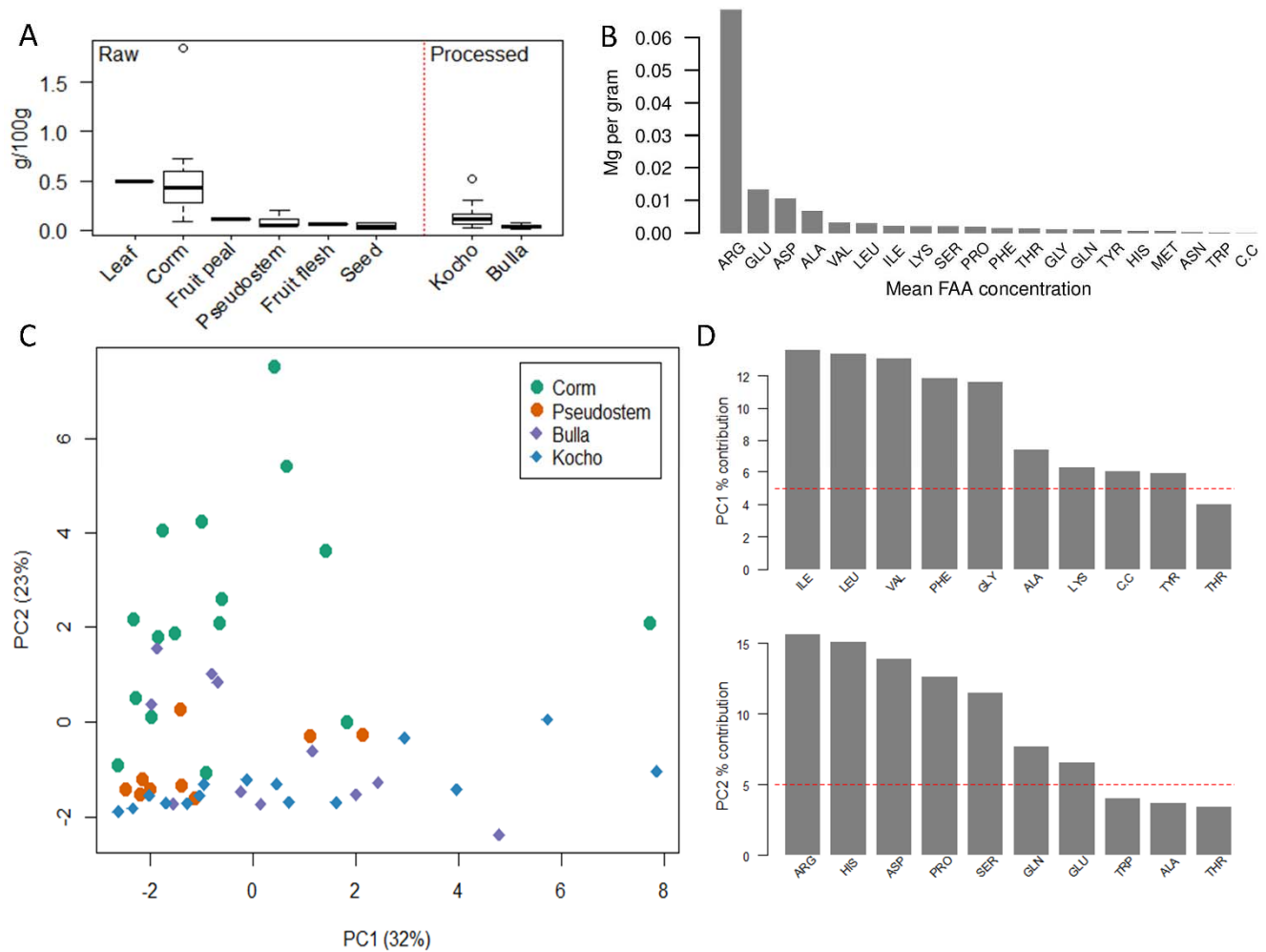
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sources and iii) Enset (pseudostem) from published sources. Comparative values for tuber and cereal

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crops are sourced from Feedipedia and the USDA Food Composition Databases.

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Figure 3. Free Amino Acid composition and variation in enset. A) Quantities of Free Amino Acids

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(FAAs) present across raw enset tissues and processed enset food products. B) Mean concentrations

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of free amino acids across all samples. C) Principal component analysis of enset FAAs across four

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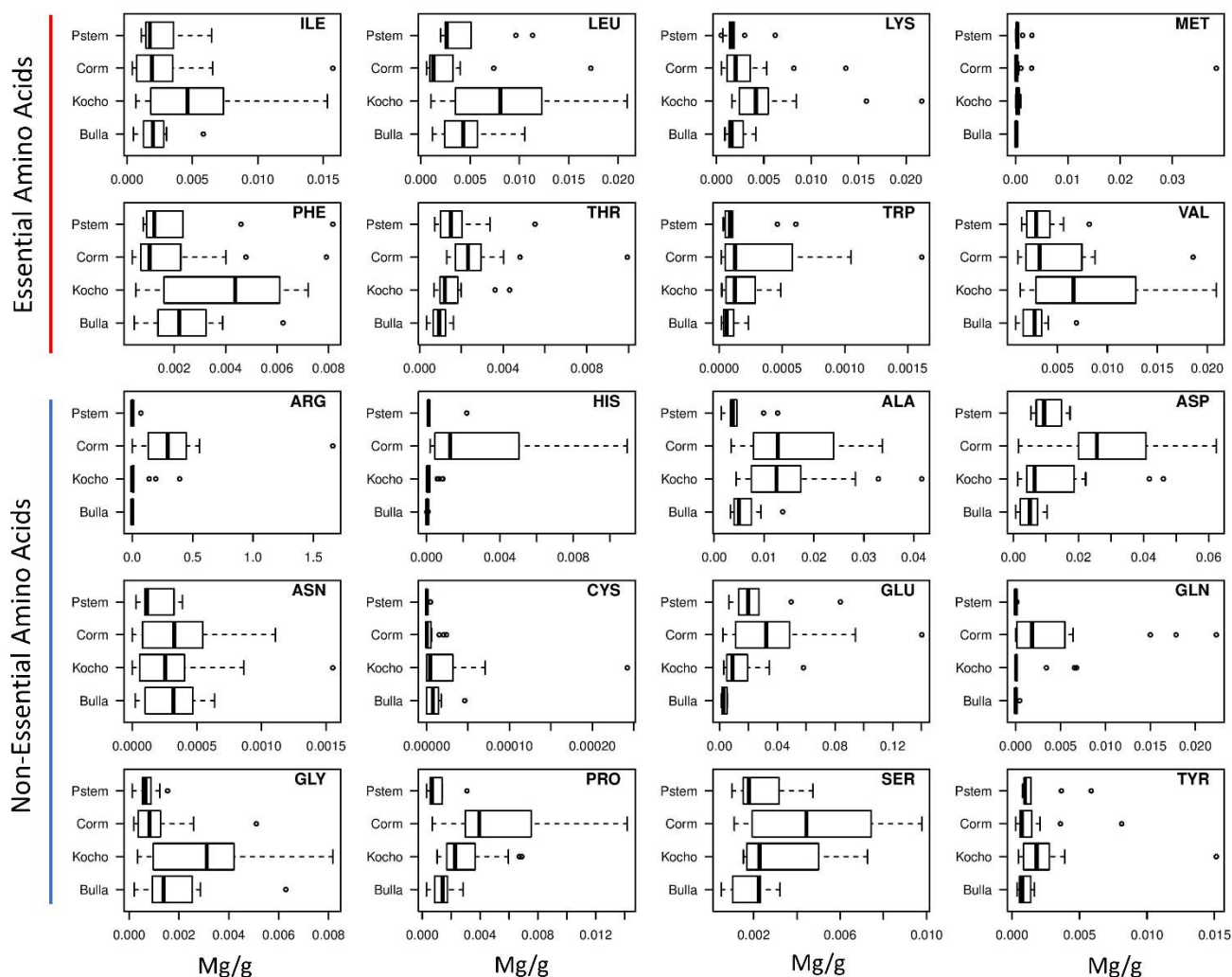
tissue types. D) Axis loading plots for the first and second principal components.

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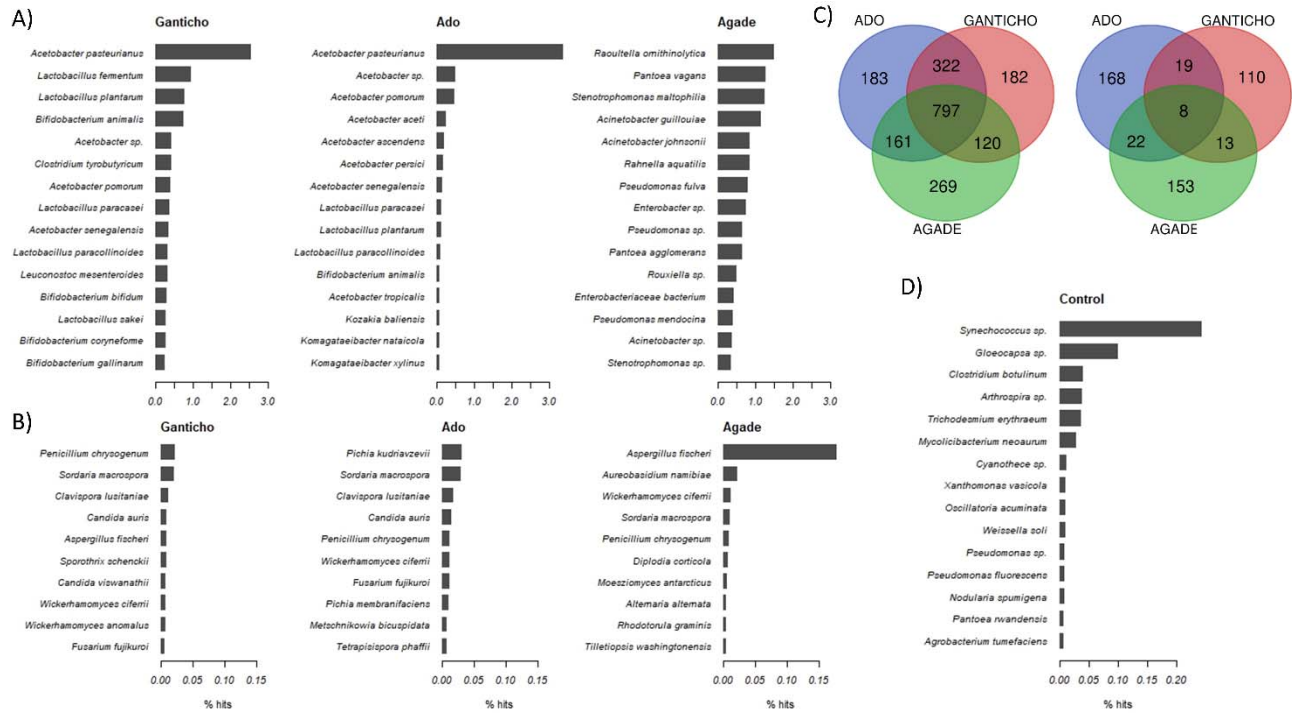
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583 **Figure 4. Free Amino Acid composition in raw enset tissues (pseudostem and corm) and processed**
 584 **food products (kocho and bulla). Whilst essential and non-essential amino acids are noted,**
 585 **conditionally essential amino acids, required in certain circumstances, are presented and include**
 586 **arginine, histidine, glycine and glutamine.**

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591 **Figure 5. Microbial composition of enset kocho samples. Bacteria (A) and Fungi (B) genomes with**
 592 **highest percentage hit rates across three kocho samples. C) Venn diagrams illustrating the number of**
 593 **species in common between samples for Bacteria (left) and Fungi (right). D) Top bacteria and fungi**
 594 **hits from enset leaf tissue**