

1 Multi-omics analyses reveal rumen microbes and secondary metabolites that are unique to  
2 livestock species.

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9 **Running title:** Rumen microbes and diet role to metabolite diversity

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22

23 **ABSTRACT**

24

25 Ruminant livestock like cattle, sheep, goat, and camel, have a unique digestive system with  
26 complex microbiota communities that facilitate feed conversion and production of various  
27 secondary metabolites including greenhouse gases, which are significant in livestock-vector and  
28 livestock environment interactions. However, there is limited understanding of the diversity of  
29 rumen microbes and secondary metabolites that have advantageous traits to livestock physiology,  
30 productivity, climate, and defense across different ruminant species. In this study using  
31 metagenomics and metabolomics data from four evolutionary distinct livestock species, we show  
32 that there are signature microbes and secondary metabolites for each species. For instance, camels host  
33 a unique anaerobic fungus(F) called *Oontomyces*, cattle harbor more unique microbes like  
34 *Psychrobacter* (F) and three unique bacteria genera *Anaeromyces*, *Cyllamyces*, and *Orpinomyces*.  
35 Goats have *Cleistothelebolus* (F), while sheep host *Liebetanzomyces* (F). This phenomenon may  
36 indicate that there are species-specific microbes that requires host rumen-microbes' environment balance.  
37 Additionally, there are conserved core bacterial microbes present and in equal abundance  
38 regardless of the host genetics, indicating their essential role in maintaining crucial functions. The  
39 studied livestock fed on diverse plant materials, including grass, shrubs to acacia trees. Regarding  
40 secondary metabolites camel rumen is rich in organic acids, goat with alcohols, and hydrocarbons,  
41 sheep with indoles and cattle with sesquiterpenes. These results have implications for manipulating  
42 the rumen environment to target specific microbes and secondary metabolite networks, thereby  
43 enhancing livestock productivity, resilience, reducing susceptibility to vectors, and  
44 environmentally preferred livestock husbandry.

45

46 **IMPORTANCE**

47 Rumen fermentation that depends on feed component and rumen microbes plays a crucial role in  
48 feed conversion and production of various metabolites, important for physiological functions,  
49 health and environmental smartness of ruminant livestock, in addition to providing food for  
50 humans. However, given the complexity and variation of the rumen ecosystem and feed of these various  
51 livestock species combined with inter-individual differences between gut microbial communities, how  
52 they influence the rumen secondary metabolites remains elusive.

53 Using metagenomics and metabolomics approaches, we show that each livestock species has  
54 signature microbe(s) and secondary metabolites. These findings may contribute towards  
55 understanding rumen ecosystem, microbiome and metabolite networks, that may provide a gateway  
56 to manipulate rumen ecosystem pathways towards making livestock production, efficient,  
57 sustainable and environmentally friendly.

58

59 **KEY WORDS** ruminants, metabolomics, rumen, fermentation, microbiota, metagenomics,  
60 metabolites

## 61 INTRODUCTION

62 Livestock are an important part of the ecosystem, especially they are a major driver in most rural  
63 landscapes, diversifying belowground microbes, soil health, function, fertility and crop  
64 productivity. Globally more than 1.2 billion people are making a living in the livestock sector  
65 across the various value chains (1, 2). Ruminant livestock provide humans with foods, such as  
66 milk and meat from non-human-edible plant material, even in arid and semi-arid ecologies, where  
67 crop production is not possible due to erratic rain fall and frequent drought, thus the only means  
68 to sustainably use such vast land is through sustainable livestock husbandry. The rumen, a large  
69 fermentation chamber in ruminant livestock, harbors diverse and complex microbial communities  
70 that play crucial roles in the digestion and fermentation of feedstuff (3, 4) and production of  
71 diverse metabolites including greenhouse gases (5, 6, 7, 8). However, livestock vary in their  
72 resilience, and feed conversion efficiency. For instance, one-humped camel (*Camelus*  
73 *dromedarius*), is the most efficient and resilient animal well adapted under scarce resources in arid  
74 and semi-arid ecologies, this is recently evidenced as pastoralists shifted from cattle to camel  
75 keeping even at higher altitudes (9, 10, 11, 12). This can be taken as a climate change adaptation  
76 strategy and has potential to improve livestock climate resilience if the underlining mechanism is  
77 understood. However, the underlying mechanisms responsible for the observed variations in  
78 resilience between different livestock is not clear. We hypothesize livestock vary in their rumen  
79 microbes and secondary metabolites that has useful traits for livestock resilience and efficiency.  
80 As rumen environment hosts the most complex diverse microbial communities consist of bacterial  
81 fungi, and protozoa etc. Therefore, understanding the diversity, pivotal role of the rumen microbes  
82 and secondary metabolites in digesting fibrous feed, providing nutrients to the host animal, defence  
83 and determining livestock host-environment interaction is key for sustainable animal husbandry.  
84 Pertinent global issues of interest include climate resilience, fight against climate change and  
85 vector borne diseases through rumen environment manipulation, to make livestock part of the  
86 solution.

87 The relationship between some members of the microbiome and rumen function is well known (5,  
88 13). The role of diet on microbes diversity has been investigated (8,14,15). Whereas host genetics  
89 have been studied in determining rumen microbes (16, 17, 18), most of the studies have been done  
90 on a single species and biased towards cattle, and no comparative studies have been reported

91 between diverse ruminant animals that vary both in feeding regime and resilience, which is the  
92 main focus of this study. Here, using four ruminant livestock that vary in feeding regime, drought  
93 resilience, and disease prevalence, we show that each livestock species created mutual association  
94 with signature microbes and secondary metabolites that provide useful ecological traits.

## 95 **RESULTS**

### 96 **Distribution of bacterial and fungal populations in the rumen**

97 To correlate the secondary metabolites with rumen microbes we performed genomic analysis of  
98 the two main rumen domains, bacteria and fungi. The taxonomic analysis of bacterial and fungal  
99 populations in the rumens of cattle, sheep, goats and camels revealed a variation in dominance of  
100 core groups of rumen microbes among the four ruminants (Fig. 1). A total of 1052 species-level,  
101 bacterial operation taxonomic units (OTUs) were uniquely identified in camels, 949 in cattle, 1065  
102 in sheep and 847 in goats respectively (Fig. 1B). Whereas 113 bacterial (OTUs) were shared by  
103 all the four ruminants, 187 OTUs were shared by both camels and goats while 208 (OTUs) were  
104 common in cattle and sheep (Fig. 1B).

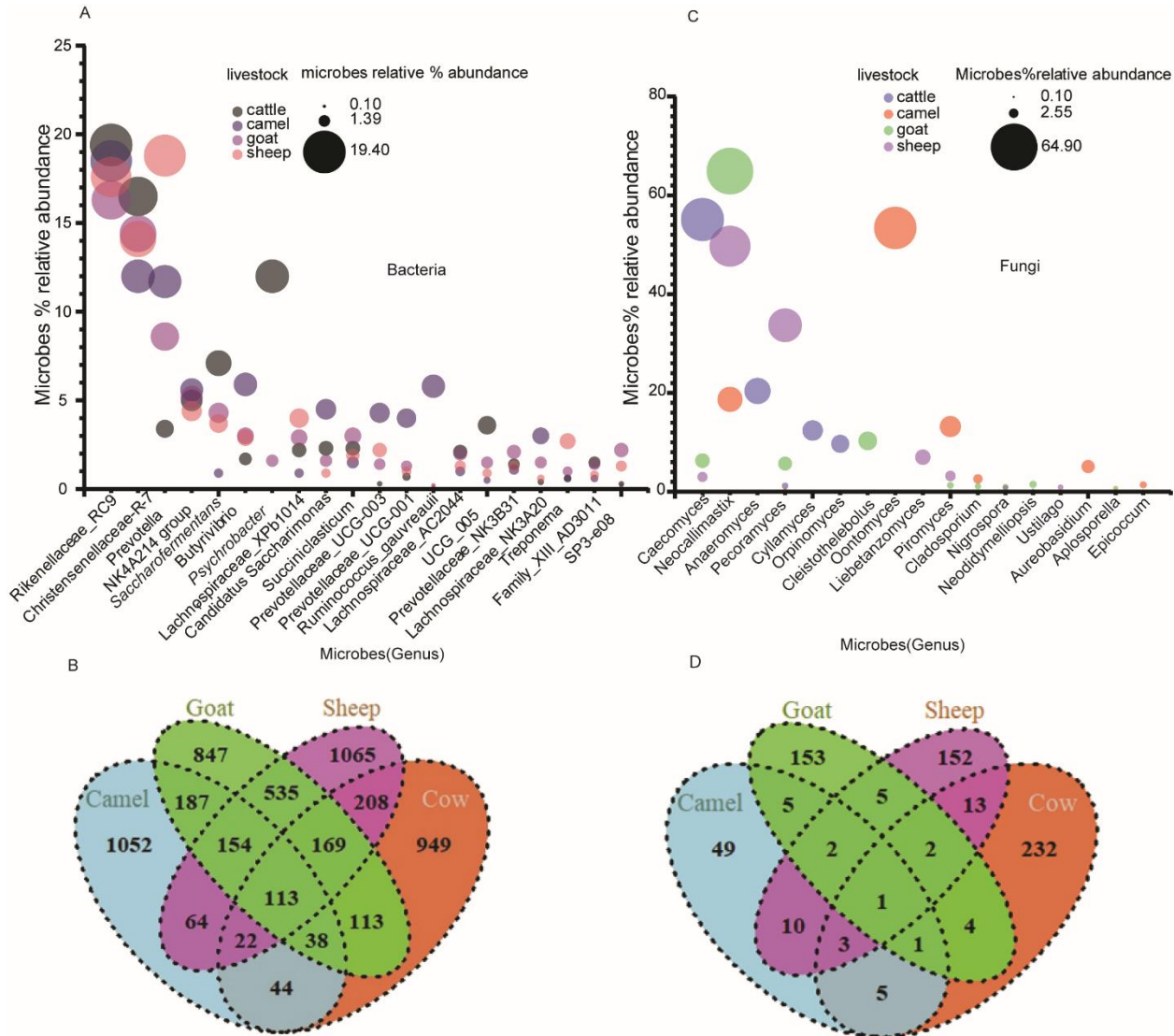
105 Bacteria being the main members of the rumen microbiome, were widely dominant across the four  
106 livestock groups analyzed, comprising most of the species richness, with some bacterial genera  
107 being livestock specific (Fig. 1A, B). A further analysis of the identified bacterial operation  
108 taxonomic units (OTUs) revealed twenty most abundant bacterial genera present among the four  
109 livestock species (Fig. 1A, C). In all four ruminants, the *Rickenellaceae RC9*, *Christensenellaceae*  
110 *R-7 group*, *NK4A214 group* and *Succiniclasicum group* are conserved both in their presence and  
111 in their high abundance (Fig. 1A). Genus *Ruminococuce* found abundantly in camels and in small  
112 amount in goats. *Prevotella*, and *Prevotellaceae* a hydrogen-producing bacterial genus, was  
113 dominant in camels, however less abundant in cattle, goat and sheep (Fig. 1A). The *Psychrobacter*  
114 genus was found uniquely in cattle and goats but dominant in cattle but absent in sheep and camel  
115 (Fig. 1A, Table S 1). All the remaining bacterial genera were conserved in all four livestock  
116 species, but with varying abundance. Compared to camels and sheep, cattle and goats had more  
117 bacterial diversity, due to an additional genus, *Psychrobacter* (Fig. 1A, Table S 1).

118

119 A comprehensive analysis was conducted on the operational taxonomic units (OTUs) of fungi in  
120 four livestock species, namely cattle, camels, goats, and sheep. The results showed a total of 232  
121 OTUs in cattle, 49 OTUs in camels, 153 OTUs in goats, and 152 OTUs in sheep at the genus level  
122 (Fig. 1C, D). Among these OTUs, a diverse population of seventeen highly prevalent fungal genera  
123 was found (Fig. 1C). The analysis further showed that only one fungal operation taxonomic units  
124 (OTUs) was common in all the four livestock species, while 5 were common in camels and goats  
125 whereas cattle and sheep shared 13 OTUs (Fig. 1D). Goat had the highest representation of fungal  
126 genera with camels having the least representation among the four ruminants (Fig. 1D). The  
127 anaerobic fungi genus *Caecomyces* was abundantly present in cattle, and in small amount in goats  
128 and sheep, but missing in camel. An aerobic fungus genus *Oontomyces* exclusively found only in  
129 camels with high abundance. *Neocallimastix* is the most abundant both in goats and sheep, present  
130 in camel in small amount, but missing in cattle. *Pecoramyces*, was found only in sheep and goat,  
131 in former much abundant, but missing from camel and cattle (Fig. 1C, Table S 2). *Liebetanzomyces*  
132 are only found in sheep. Furthermore, *Anaeromyces*, *Orpinomyces* and *Cyllamyces* were unique to  
133 cattle, whereas *Piromyces* were the major groups in camels (Fig. 1C, Table S 2). *Nigrospora* is  
134 absent in cattle, but present in the other three livestock in small amount. *Caecomyces* was dominant  
135 in cattle, absent in camel, but present in small amount in goat and sheep. *Cleistothelebolus* was  
136 distinct to goats, may be considered as signature fungal community in goat rumen (Fig. 1C, Table  
137 S2). Only *Cladosporium* and *Pecoramyces* are conserved among the four livestock demonstrating  
138 their requirement for conserved function. Furthermore, camel harbour different protozoans as  
139 compared to other livestock (data not shown).

140

141



142

143 Fig. 1: Bacteria and Fungi community compositions in different livestock

144 A bubble plot showing the qualitative and quantitative difference of bacteria, plot generated using

145 bacteria relative abundance data with at least 1% relative abundance in one of the four livestock

146 species. (B). Venn diagrams showing the identified bacterial Operational Taxonomic Units

147 (OTUs) unique and shared between livestock species (C) A bubble plot showing the qualitative

148 and quantitative difference of fungi with at least 1% relative abundance in one of the four livestock

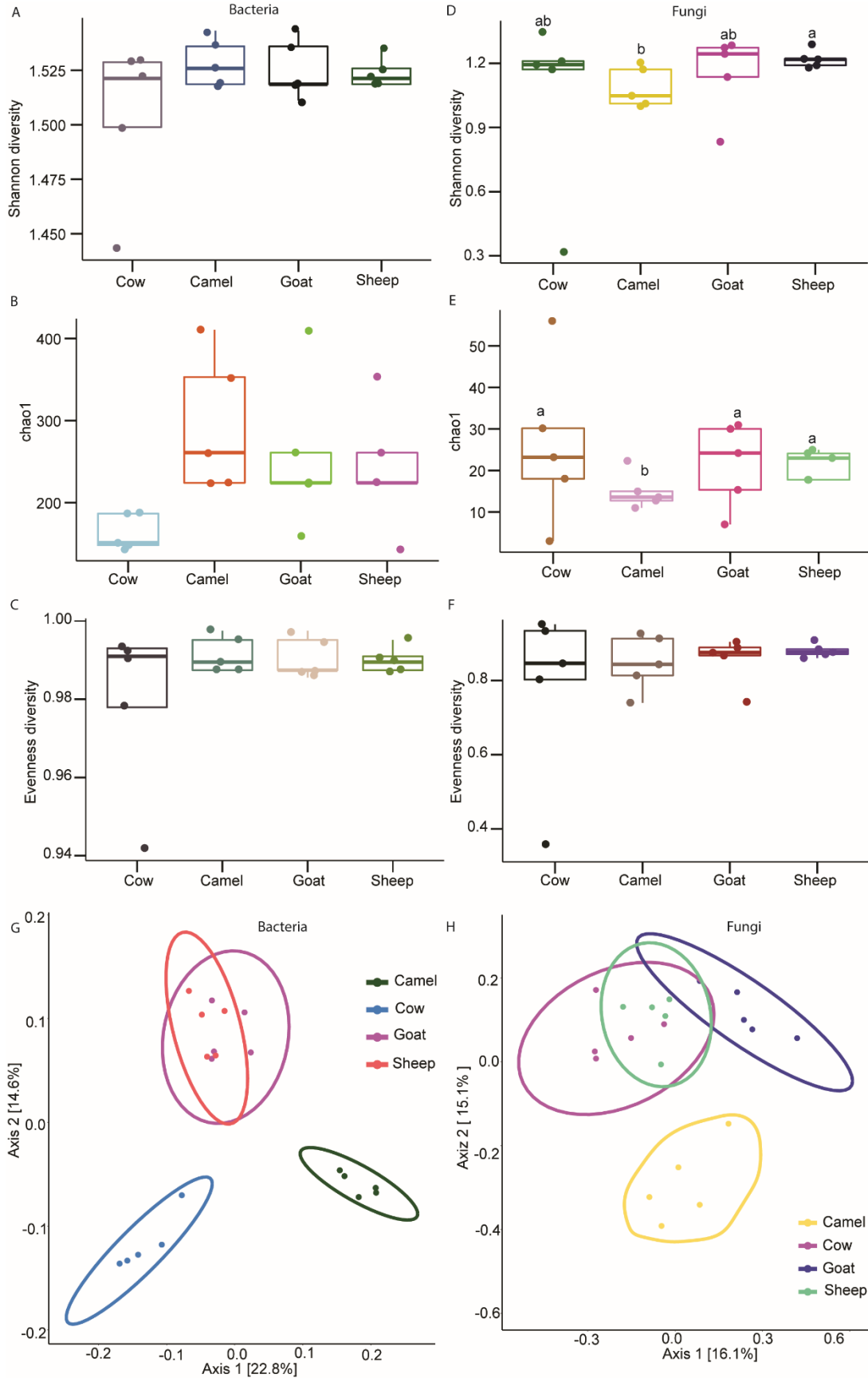
149 species. (D) Fungal Operational Taxonomic Units (OTUs) among different livestock.

150

151 **Alpha and Beta Diversity**

152 The four ruminants showed greater variability and diversity in bacterial and fungal populations as  
153 revealed by Shannon, Chao1 and Pielou evenness, alpha diversity indices (Fig. 3A-F). However,  
154 cattle and goats showed similarity in evenness and richness. Beta diversity was assessed by  
155 calculating the PCoA of different rumen bacterial and fungal domains using Bray-Curtis method.  
156 The analysis revealed significant dissimilarities in bacterial and fungal domains distributions  
157 among ruminants as displayed by the different eclipse clusters ( $p = 0.004$ , PERMANOVA; Fig.  
158 2G-H). Compared to other ruminants, cattle and camels showed higher variability of bacterial  
159 communities (Fig. 2G), whereas camels exhibited a different fungal domain population (Fig. 2H).



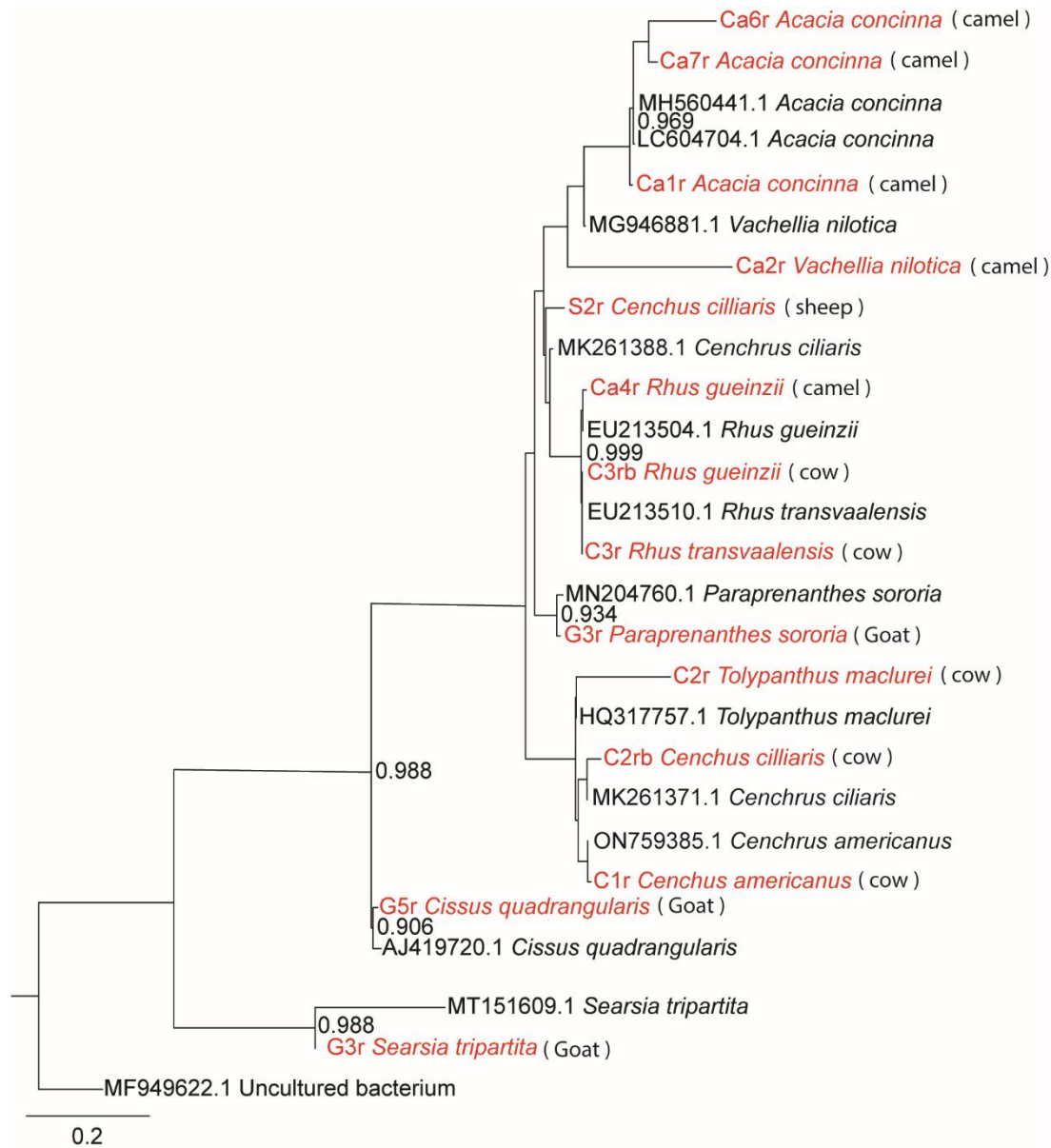


161 Fig. 2: Alpha (A-F) diversity indices for bacterial and fungal populations using Shannon index ( $p$   
162 = 0.77, and 0.28; A and D). Chao1 richness index estimates of bacteria and fungi ( $p =$ , 0.036, 0.24;  
163 B and E). Evenness estimates in bacteria and fungi ( $p =$  0.87, 0.95; C and F). Beta diversity PCoA  
164 ellipse clusters based on unweighted unfrac distance dissimilarity method showing the distribution  
165 of bacteria and fungi (G-H). Bars followed by different letters are statistically significant.

166

167 **Dietary composition assessment in livestock rumen**

168 Microbial diversity and secondary metabolites may be affected by host diet composition beside  
169 host's individual genetic makeup (8). To understand the overall metabolites make up in relation to  
170 diet among ruminants, the study characterized the various diet consumed by the four livestock. In  
171 pastoralist setup where feed is not controlled or restricted, livestock can feed on a wide range of  
172 plant materials. For instance, we found that in addition to grasses (*poaceace*), *Cenchrus ciliaris*  
173 and *Cenchrus americanus*, which had been consumed by cattle and sheep, cattle had consumed  
174 other plant species such as *Rhus gweinzii* and *Rhus transvaalensis* despite being predominantly  
175 grazers (Fig. 3). Unlike cattle and sheep, camels and goat are specially adapted to feed on leaves,  
176 fruits of high-growing woody plants, soft shoots and shrubs, such as *Acacia concinna*,  
177 *Paraprenanthes sororia*, *Vachellia nilotica* and *Searsia tripartita* (Fig. 3), which are  
178 predominantly found within arid and semi-arid areas. Therefore, points to the diversity in dietary  
179 composition among the ruminants, which influences both the metabolite compound and microbial  
180 population composition among the ruminants.



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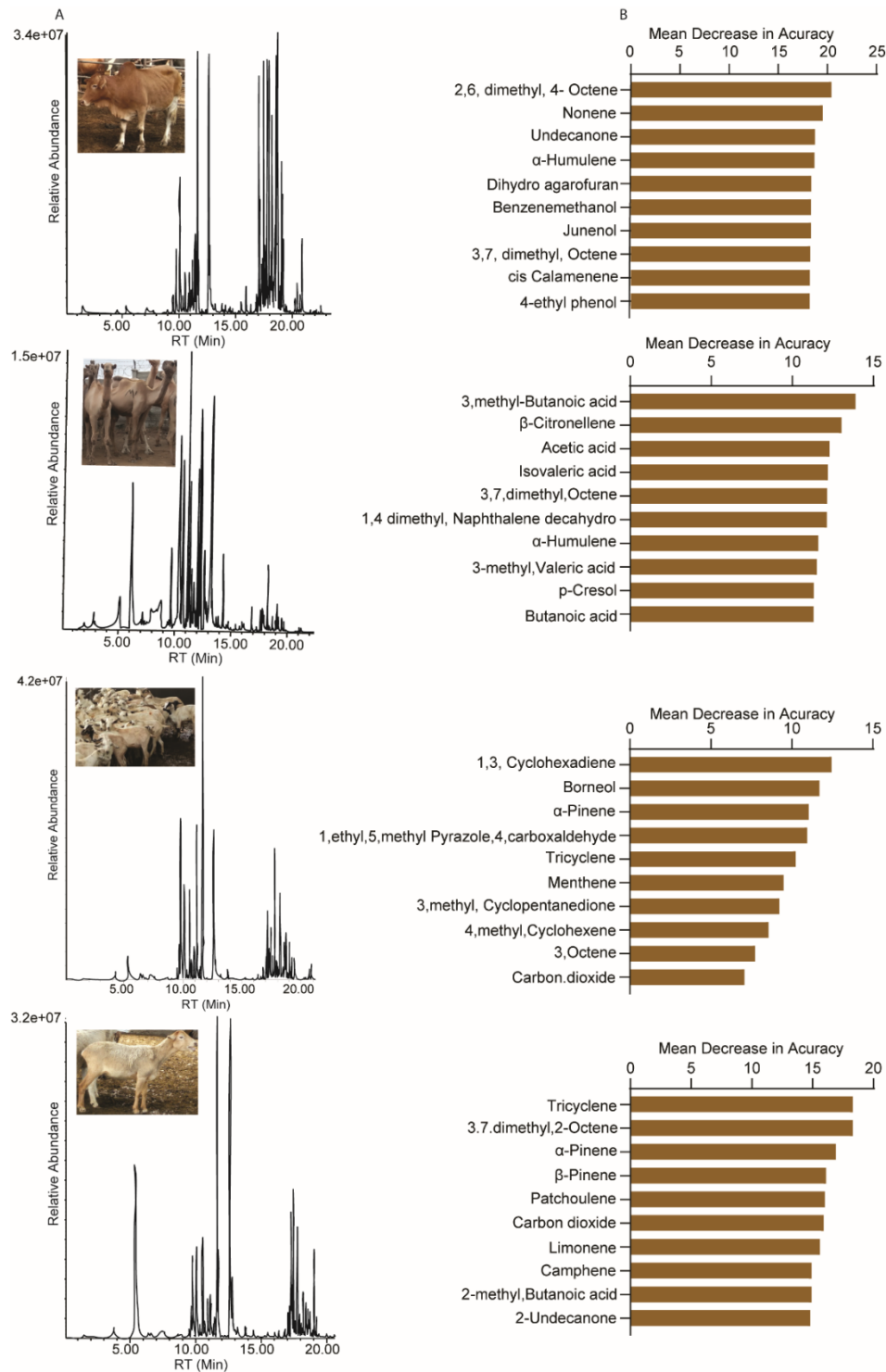
182 Fig. 3: Phylogenetic tree showing plant diet composition identified in livestock rumens, analysis  
 183 based on maximum likelihood, branch values indicate bootstrap support of 1000 pseudo replicates.

#### 184 **Ruminal metabolite composition in livestock**

185 In the present study, a total of 162 metabolite compounds (Table S 3) were identified in bovine  
 186 rumen content of four livestock; cattle, sheep, goat and camel by GC-MS. The detected  
 187 compounds represented various chemical classes, including alcohols, ketone, phenols, volatile  
 188 fatty acids, terpenes, esters and hydrocarbons. Although most major classes of secondary  
 189 metabolites have ubiquitous distributions among the four livestock, each livestock species has its

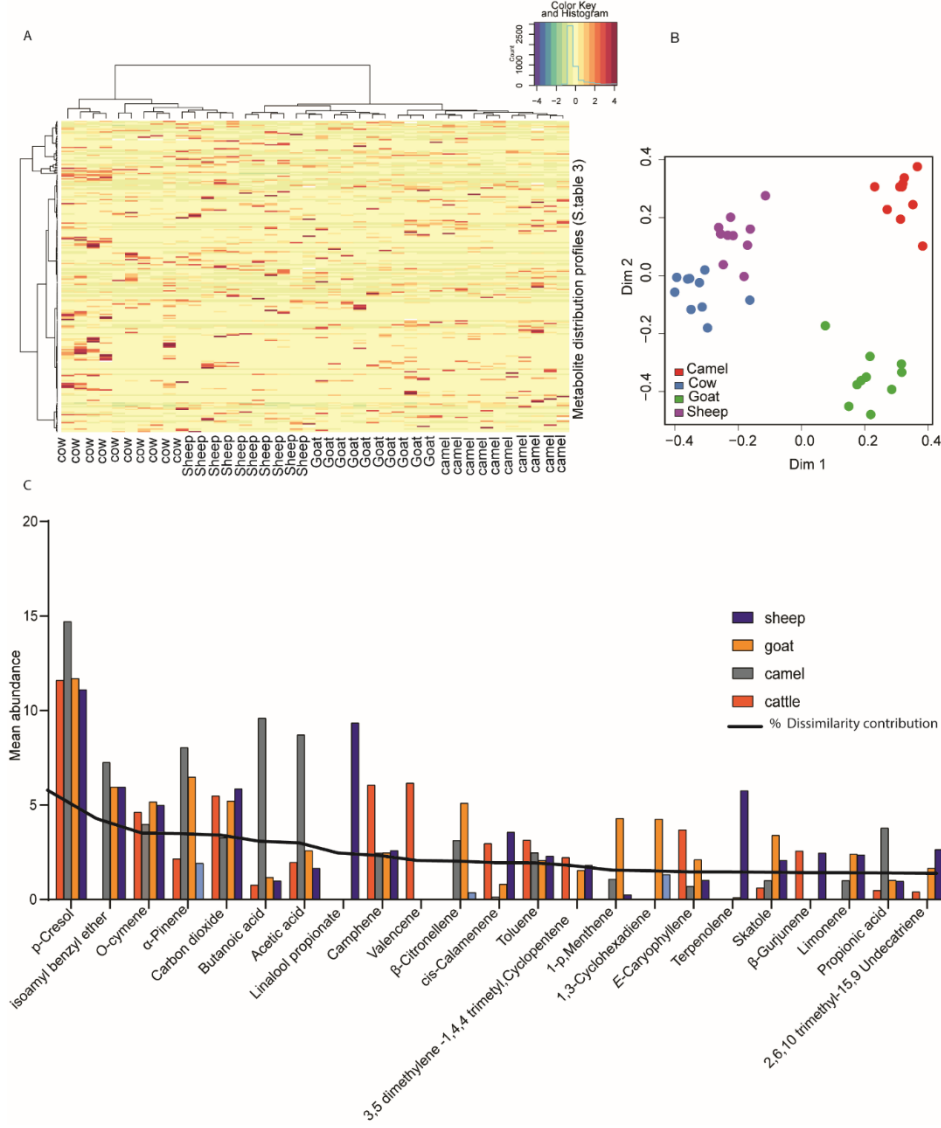
190 own signature secondary metabolites (Fig. 4A). A random forest classification was conducted to  
191 reveal the top 10 predictive compounds for individual ruminant species (Fig. 4B). 2, 6 dimethyl,  
192 4-octene, 3 Methyl, butanoic acid, 1, 3 cyclohexene and tricyclene being the most predictive  
193 secondary metabolite compounds of cattle, camels, goats and sheep rumen, respectively. In camel  
194 5 out of the ten predictive compounds are acids, signifying the diversity of acid in camel rumen.  
195 However, we did not observe dominance of any specific chemical class in the other livestock,  
196 diverse class of compounds contribute for predictive signature odours. The diversity and contrast  
197 in metabolite composition among the ruminants was revealed by the clustering and segregation of  
198 the respective species based on their metabolite composition by multidimensional scaling (MDS)  
199 and matrix plot (Fig. 5A-B). While some species such as cattle, and sheep, which are grazers  
200 clustered in close proximity, camels and goats, which are browsers, were distinctly clustered apart  
201 from the other ruminants and also from each other (Fig. 5B). Thus, demonstrating a similarity in  
202 metabolite composition among grazers (cattle and sheep) and but not clear with browsers (camels  
203 and goats). Overall, the four livestock dissimilarity based on their rumen secondary metabolites  
204 was 72.5%. Twenty-three compounds, based on quantitative and qualitative difference contributed  
205 for more than 50% of the variation (Fig. 5C). Acids are found in most abundant in camel, the three  
206 livestock produced almost 2x carbon dioxide as compared to camel. Isoamyl benzyl ether absent  
207 in cattle, linalool propionate detected only in sheep, valence detected only in cattle, citronellene-  
208 beta absent in cattle, cis-calamine absent in camel, menthane-1-p absent in cattle and sheep, 1, 3,  
209 cyclohexadiene found only in goat and sheep, terpinolene found only in sheep,  $\beta$ -gurjunene absent  
210 in camel and goat, limonene absent in cattle, 1, 5, 9-undecatriene absent in camel.

211



212

213 Fig. 4: GC-MS chromatogram profiles of metabolite compounds in ruminal fluid of various  
 214 livestock (A), cattle, camel, sheep and goat respectively. Histograms showing the classification of  
 215 the top ten predictive compounds from different livestock rumens based on their Mean Decrease  
 216 in accuracy (MDA) of the Random Forest analysis (B).



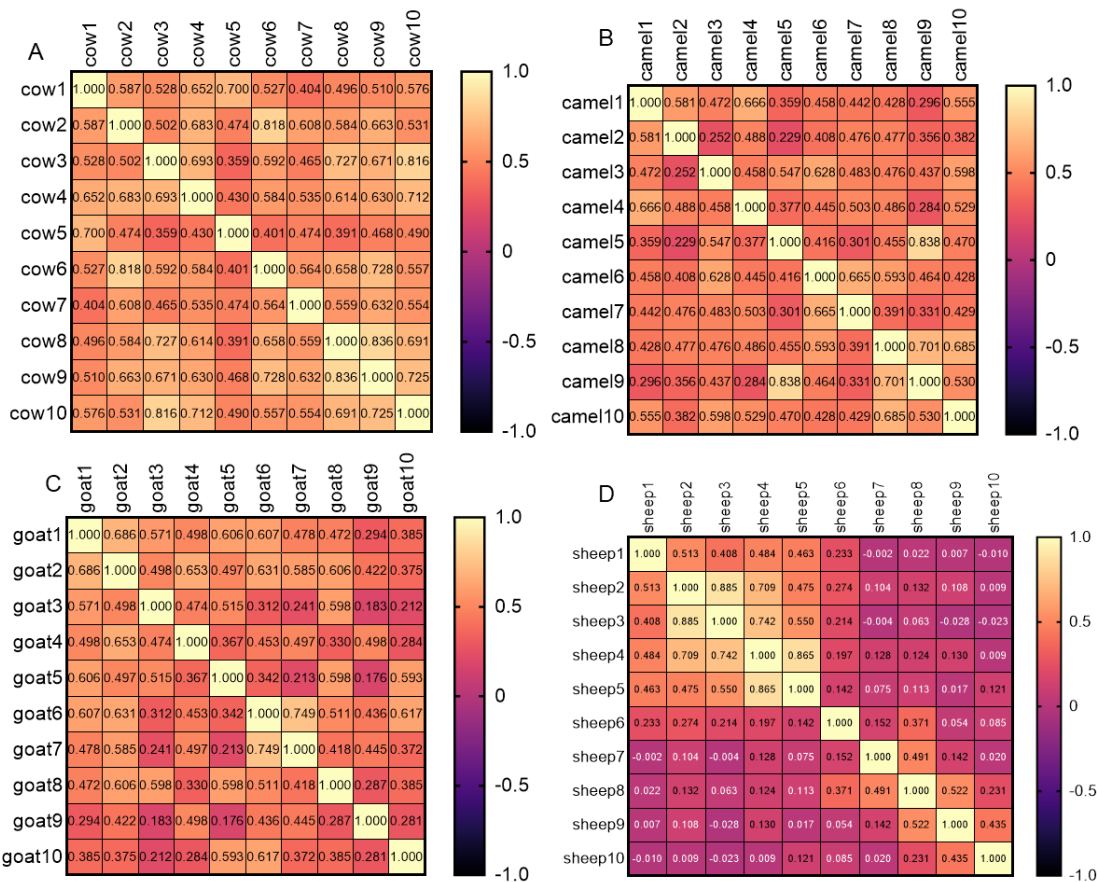
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218 Fig. 5: Heatmap coded matrix showing relative percent contribution of individual compounds to  
 219 the total composition of each livestock species (A) (for detail please see (Table S 3).  
 220 Multidimensional scaling (MDS) plot showing the segregation of ruminants based on metabolite  
 221 composition (B). Histogram showing the classification of the top twenty-three for 50%  
 222 dissimilarity contributing metabolite compounds from all livestock based on SIMPER analysis,  
 223 the line graph shows the percentage contribution of a given compound for the dissimilarity. (C).

224

## 225 Variability in metabolite composition among individual species population

226 The correlation between populations of the same species dynamics and the metabolite compound  
 227 profiles of four types of livestock was investigated using a Pearson's correlation analysis.  
 228 Generally, a minimal variability in metabolites was observed between individual of the same  
 229 species. Cattle, goats, and camels showed minimal variability in their volatile organic compound  
 230 profiles (Fig. 6C); however, sheep populations showed some variation in sheep 7, 8, 9, and 10  
 231 (Fig. 6D). As a result, the rumen odor profiles in the herd populations of the four cattle species  
 232 used in this investigation were comparable.



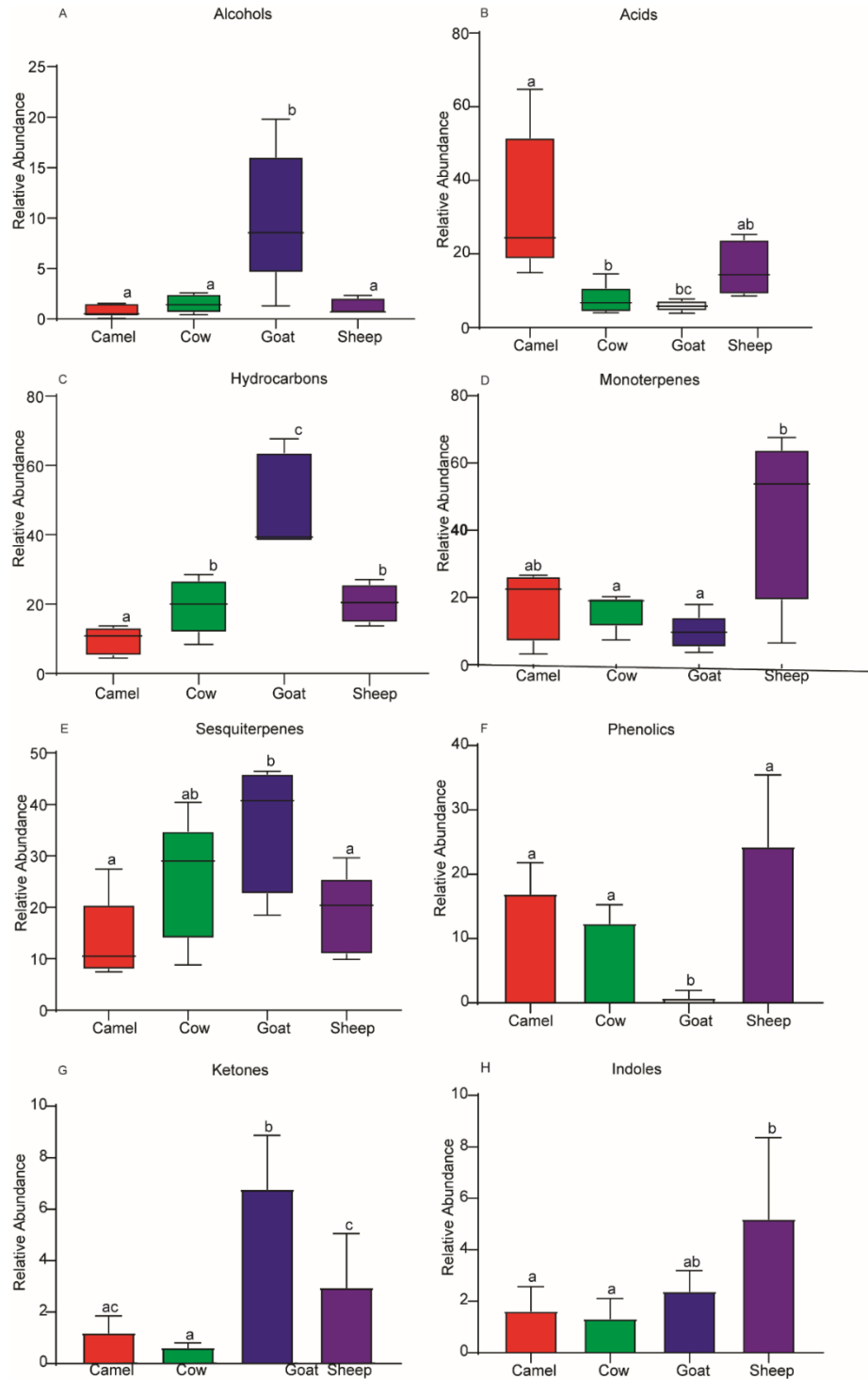
233  
 234 Fig. 6: Color coded Pearson's correlation plots for identified volatile organic compounds among  
 235 individual animal in their respective species.

236



## 237 **Metabolite composition by chemical functional groups**

238 We then evaluated the chemical identities and variation in distribution of volatile organic  
239 compounds across the four ruminant species, with compounds categorized based on their  
240 functional group classification, including phenols, alcohols, indoles, monoterpenes,  
241 sesquiterpenes, acids, hydrocarbons, and ketones. We found significant differences in the relative  
242 abundance of certain chemical class, such as alcohols, hydrocarbons, monoterpenes, acids, and  
243 sesquiterpenes, among the four livestock groups, with cattle, sheep, camel, and goats displaying  
244 varying relative abundance of these compounds (Fig. 7A-G,  $P < 0.05$ ). Cattle, sheep, and camel  
245 had significantly lower alcohols and sesquiterpenes concentrations compared to goats (Fig. 7A &  
246 E, ANOVA,  $P < 0.05$ ). Similarly, acids varied between the four ruminants, with camels having  
247 significantly high acid abundance compared to cattle, sheep and goats (ANOVA,  $P = 0.004$ ) (Fig.  
248 7B), that resulted in acidic rumen environment in camel. A significant variation was noted in camel  
249 ruminal pH compared to the other three livestock groups (ANOVA,  $P < 0.05$ ). Cattle, goat and  
250 sheep had a relatively neutral pH ranging from (7.0 -7.4), compared to camel which had a acidic  
251 pH (pH 6.3-6.5). Phenols, ketones, and hydrocarbons are more abundant in goats compared to the  
252 other livestock, sheep has more indoles compared to other livestock (ANOVA,  $P < 0.05$ ) (Fig. 7H).



253

254 Fig. 7: Box plots showing variation of chemical families of identified metabolite compounds  
255 among different ruminants (A-H). Bar graphs followed by different letters are statistically different  
256 in their abundance.

257

258 **DISCUSSION**

259

260 In this comparative study we show that there is a complex network of rumen microorganisms that  
261 coexist, interact and compete for substrates resulting in a critical balance of various end products  
262 such as secondary metabolites without autotoxicity to provide energy for microbial growth, and  
263 beneficial end products for the host. A better understanding of how the rumen microbiome  
264 influences host health and performance may lead to novel strategies and treatments for trait  
265 improvement in the livestock sector in nature inspired ways.

266

267 Three bacterial genera (*Rikenellaceae RC9 gut group*, *Prevotella*, *NK4A214 group* and  
268 *Christensenellaceae R-7 group*) were highly conserved both in their presence and abundance  
269 among the four livestock species regardless of genetics which may suggest that they are core rumen  
270 bacteria, essential for highly conserved common traits or function for these animals. However,  
271 there were unique bacteria in a given host, for instance *Anaeromyces*, *Cyllamyces* and  
272 *Orpinomyces* found only in cattle, which may be suggestive of the existence of species specific  
273 microbes that requires host rumen-microbes' environment balance. Similarly, two fungi genera  
274 *Cladosporium* and *Pecoramyces* are conserved between the four livestock in their presence, but  
275 not in abundance demonstrating they are necessary for a conserved function, and their function  
276 may be relative abundance dependent in each species. Several fungi genera are unique either  
277 present only in one animal species or shared between some of them but not by all the four, even  
278 those that are shared present in different abundance, that may be integral to their environment and  
279 potentially compatible with rumen environment and host requirements. The significant variation  
280 in fungi microbes between the four rumen livestock may be explained to a significant extent by  
281 host genetics (5, 16, 17, 18). This unique microbes-host framework variation in microbial  
282 composition between hosts may affect microbially-mediated ecosystem processes as well as may  
283 depend on host phylogenetic relatedness and trait-based patterns of ecologies (19). Each of the  
284 bacterial and fungal communities established in the present study, play a specific metabolic role  
285 in the rumen (20, 21, 22). For instance, bacterial species like *Ruminococcus*, *Lachnospiraceae*,  
286 *Christensenellaceae* and *Prevotella* are associated with hydrogen production during rumen  
287 fermentation (23, 24). There are some microbes that were ubiquitous in all four rumen species

288 demonstrating their wide rumen environment adaptation, for instance camel rumen has acidic pH  
289 compared to other livestock.

290 The next step is to explicitly link the observed microbial, secondary metabolites diversity and  
291 network with basic evolutionary principles, which is biological fitness. We have shown the  
292 variation between diverse microbes among the four livestock that varies in feeding behaviour,  
293 draught resilience and disease susceptibility (25). For instance, camel and small ruminant to some  
294 extent are the most resilient among analyzed livestock to frequent drought as compared to cattle  
295 (9,10,11,12). This could be due to the abundantly presence of unique anaerobic fungi, *Oontomyces*,  
296 originally identified from Indian camel (26) and bacteria (*Prevotella*) in camel that have  
297 demonstrated high capability of diet conversion (27, 28, 29, 15). Additionally, fungus  
298 *Neocallimastix*, which is present in camel, sheep and goat but absent in cattle, have been shown  
299 to be effective in bioconversion potential of poor diet such as lignocellulose into useful products  
300 (30, 31) may have contributed for their resilience. These microbes combine with other  
301 physiological mechanism such as suppression of cholesterol biosynthesis in kidney of camel to  
302 retain and reabsorption of water (32) may contributed for camel draught resilience. Thus, camel's  
303 evolutionary success to dry climate, partly may be due to the ability to engage in mutualistic  
304 interactions with useful microbes that provide novel ecological adaptation traits. Furthermore, such  
305 knowledge will give us opportunity to manipulate rumen environment to make livestock less  
306 susceptible to vectors, efficient in converting their diet to animal protein and to make livestock  
307 environmentally friendly. For instance, in one study, the addition of a fungal inoculant to the diet  
308 of dairy cows was found to increase the production of propionate and decrease the production of  
309 acetate (33), which is a precursors of greenhouse gas production. Furthermore, microbiome work  
310 in humans and rodents has revealed that microbes play essential roles in host health and function  
311 (34, 35). Similarly, in our previous work these various livestock exhibited various susceptibility  
312 to various pathogens (36) that may depend on their mutualistic association with useful microbes.

313  
314 The study conducted by (5) shown that the core microbiome had a significant explanatory role in  
315 relation to dietary components within a controlled experimental setting. In our experiment, it was  
316 difficult to dissect the role of diet for the microbes and chemodiversity variation as the animals  
317 were from free grazing and browsing set up and fed on diverse diets. If we assume that diet may  
318 structure rumen microbes we would have expected similarity both in microbes and secondary

319 metabolites between browsers (camel and goat) and also between grazers (cattle and sheep).  
320 However, we did not find a clear link between diet and microbes, for instance only one bacterial  
321 genera *Psychrobacter* is missing in camel and sheep. If diet shape the rumen microbes, browsers  
322 (camel and goat) should share more similar microbes than camel has in common with cow and  
323 sheep and vice versa. On the other hand, if microbes dictate diet, cattle and sheep share more  
324 similar microbes than what camel and cattle share. But sheep and goat shared more bacteria than  
325 either of them shared between camel and cattle. This may also be because there is no strict browsers  
326 and grazers under free grazing setting as they can easily shift between various diets depending on  
327 feed availability. The various plants consumed by the various livestock are characterized by high  
328 fiber content, and rich in secondary metabolites, and bioactive compounds including tannins,  
329 flavonoids, alkaloids, and terpenoids which may have potential health benefits for ruminants (37,  
330 38, 39). The utilization of shrubs and woody plants in livestock diets has been shown to increase  
331 rumen metabolite richness compared to diets based on traditional forage sources (40, 41, 42).  
332 Studies showed that feeding goats on *Acacia saligna*, a shrub species, led to increased diversity  
333 and richness of rumen metabolites compared to a control diet based on alfalfa hay (40, 41, 42, 43).  
334 The composition of the plant diet can have significant impacts on the production of metabolites in  
335 the rumen for instance, Grasses (*Poaceae*) contain fermentable cellulose, hemicellulose, lignin,  
336 and protein which are broken down by rumen microbes into various metabolites, including acetate,  
337 propionate (13, 44), which are ingredients in greenhouse gas formation and energy source. Hence  
338 the variability of plant diets can have a significant impact on rumen metabolite production and  
339 composition in livestock.

340 Rumen fermentation is a complex process that results in the production of various metabolites (5,  
341 33). We established a wide range of secondary metabolite compounds in rumen, which is an  
342 interplay between, host genetics, diet and microbes, most of which are associated with various  
343 biochemical activities in livestock rumen. The detection of metabolite compound classes, such as  
344 volatile fatty acids, aromatic hydrocarbons, terpenes, hydrocarbons, phenols, and alcohols,  
345 displays the diversity and complexity of metabolic synthetic pathways in livestock rumens,  
346 leading to the production of several diverse metabolites (45). The detection of plant-derived  
347 metabolite compounds such as camphene,  $\alpha$ -pinene, and  $\beta$ -caryophyllene, including fecal  
348 predictive indolic and phenolic compounds like p-cresol (a byproduct of protein breakdown in  
349 animal gut) and skatole, which had previously been reported in various animals metabolic by

350 products, for instances in animal feces, which have role in livestock-vectors interaction (45, 46,  
351 47, 48), demonstrate that metabolites are conserved as they pass through various digestion process.  
352 But we also observed less complexity in some metabolites for instance, phenols in the rumen are  
353 less complex as compared to livestock urine (36), demonstrating metabolites may gain complexity  
354 after they left rumen.

355 Even though the examined metabolite composition varied among the ruminants, minimal  
356 intraspecific variation was realized among individual species herd, indicating a potential host  
357 specific microbes and host genotype effect (49) implies that rumen secondary metabolites may not  
358 be affected by livestock population dynamics as was demonstrated by (36, 50). Despite their  
359 diversity among the 4 ruminants, identical metabolite compound classes were detected from rumen  
360 metabolism, point to a similarity in their biosynthetic pathways between the four livestock species  
361 and those metabolites may have conserved function regardless of the host genetics.

362  
363 Studies have highlighted a direct relationship between bacterial and fungal populace with rumen  
364 metabolome (51, 52, 53, 54, 55, 56). These microorganisms work together in a symbiotic  
365 relationship with the host to break down complex plant polysaccharides and fiber into simple  
366 sugars, which can then be fermented into volatile fatty acids (VFAs), microbial proteins and other  
367 metabolites that can be absorbed by the host animal (8, 57). Thus, the various secondary  
368 metabolites identified may provide various functions to the host. Ruminant, such as cattle, sheep,  
369 and goats utilize hydrocarbons as energy source largely contained in plant carbohydrates like  
370 glucose and sucrose, by fermenting them in their rumen into volatile fatty acids, which are then  
371 absorbed and utilized for energy (58, 59). In this study, we established notable differences in  
372 hydrocarbons, terpenes, ketones, and indoles relative abundance among the ruminants. Such  
373 variations clarify relevant aspects such as diet composition, breed, and environment, since the  
374 detection and concentration of most ruminal metabolite compounds are influenced by these factors  
375 (60, 61, 62). The diversity and importance of different compound classes of rumen metabolome in  
376 livestock were further demonstrated by the detection of terpenes, which have been linked to  
377 improve nutrient utilization and digestive health (63). In addition to terpenes, chemical compounds  
378 classes like acids, phenols, indoles, ketones, and alcohols, also varied significantly among the  
379 ruminants (Fig. 7).

380 Acids profile significantly differs between the four livestock species, being the highest in camel.  
381 Acids are involved in the hydrolysis of complex carbohydrates, such as cellulose, lignin and  
382 hemicellulose, into simpler sugars that can be further metabolized by rumen microbes (64). This  
383 may be ascribed to the fact that acids are energy sources for the host animal and can be used as  
384 precursors for energy production during special conditions. For instance, fatty acids such as acetate  
385 is used by the host animal as a precursor for fatty acid synthesis in adipose tissues, which can then  
386 be utilized as an energy source during times of high energy demand, such as during lactation or  
387 periods of feed restriction (65), thus the diverse acids produced in camel rumen may have  
388 contributed to camel rumen acidic pH, resilience even during extended drought, with limited feed  
389 availability in arid and semi-arid ecologies. Phenols and indoles are aromatic compounds that are  
390 derived from lignin, which is present in the cell wall of plants, are produced during the  
391 fermentation of plant material in the rumen, and have been shown to have antimicrobial properties  
392 that can help to maintain a healthy microbial balance in the rumen (66), and antioxidants, which  
393 can help to reduce oxidative stress in the rumen and improve animal health (67, 68). Alcohols,  
394 provide energy for rumen microbes in addition to being a carbon source for the synthesis of  
395 microbial protein (69) and ketones shown to be an alternative energy source for ruminants in  
396 addition to preventing ketosis (70). Furthermore, elucidation of maternal, genetic, and  
397 environmental factors, rumen environment (for instance pH, nutrient etc) that influence rumen  
398 microbiome establishment and development may provide novel insights into possible mechanisms  
399 for manipulating the rumen microbial and secondary metabolites composition to enhance long-  
400 term host health, performance and climate resilience.

401

## 402 **Conclusion**

403 We have documented various microbes and secondary metabolites which vary among ruminants that  
404 may provide, useful traits, such as energy source, antioxidant, digestive and detoxifying  
405 capabilities, improve host defense against pathogens. We can conclude the diversity both  
406 qualitative and quantitative in microbes may contribute to the variation observed between the four  
407 livestock phenotypic traits expressed by the host animal including chemodiversity and resilience.  
408 Our result may have application in rumen environment manipulation targeting microbes and  
409 secondary metabolites network to make livestock productive, resilient, and less susceptible to

410 vectors and environmentally preferred, climate smart livestock husbandry. Our results demonstrate  
411 rumen fermentation at the interface of host genetics, microbes and diets has a significant  
412 implication for the production of complex secondary metabolites, which in turn can confer unique  
413 ecological traits to the host organisms.



## 414 **MATERIALS AND METHODS**

### 415 **Collection of rumen content**

416 Bovine rumen contents were collected from 10 different freshly slaughtered boran cattle (*Bos*  
417 *indicus*), goats (*Capra aegagrus hircus*), sheep (*Ovis aries*) and camels (*Camelus dromedaries*)  
418 from their respective abattoirs in Nairobi and Machakos County, respectively. The samples (500ml  
419 each) were kept in sterile airtight freeze-resistant 1L odor collection glass jars (Sigma Scientific,  
420 USA) and transported in a cooler box to the laboratory for metabolite compound collection and  
421 analysis.

422

### 423 **Genomic DNA extraction**

424 To extract genomic DNA from rumen contents of cattle, sheep, camels, and goats, 200 µl of the  
425 sample was mixed with an equal volume of buffered phenol and 20 µl of 20% SDS in a 2 mL  
426 centrifuge tube (Eppendorf, Germany). After adding 0.5g of 2 mm zirconia beads (BioSpec Inc.,  
427 USA), the mixture was shaken thrice in a mini-tissue lyser (Qiagen, Hilden, Germany), at a  
428 frequency of 30Hz for 90 seconds. The lysate was then centrifuged at 14000 rpm on a 5417R  
429 centrifuge (Eppendorf, Germany) for 10 minutes, and the supernatant was transferred to a 1.5ml  
430 clean tube (Eppendorf, Germany). Afterwards, 200 µl of buffered phenol was added to the  
431 supernatant, the mixture was briefly vortexed, and then centrifuged at 14000 rpm at 4°C for 15  
432 minutes. The DNA was then precipitated by adding 500 µl absolute ethanol to the supernatant in  
433 a clean 1.5ml centrifuge tube and centrifuged at 14000 rpm at 4°C for 5 minutes. The supernatant  
434 was discarded, and DNA pellet washed by 500ul of 70% ethanol then centrifuged for 5 minutes.  
435 Finally, the pellet was suspended in 100 µl of preheated elution buffer G (ISOLATE II Genomic  
436 DNA kit, Bioline Meridian). The DNA quality and quantity was checked by Nanodrop  
437 spectrophotometer (Thermo Scientific, Wilmington, DE, United States). Aliquots of 50µl of the  
438 obtained DNA extracts were sent to Macrogen Inc (Netherlands) for Illumina next-generation  
439 sequencing (NGS) targeting 16S rRNA and ITS1 for bacteria and fungi respectively. The  
440 remaining amounts (50µl) were utilized for PCR for plant diet identification.

441

## 442 **PCR amplification for diet composition screening**

443 PCR amplification targeting two chloroplast markers, consisting of coding (rbcL gene) and non-  
444 coding gene spacer region (trnH-psbA) primers (Table S 4) was done according (71). The obtained  
445 amplicons were then sent for sequencing at MacroGen Inc (Netherlands). Using Geneious software,  
446 obtained sequences were cleaned, edited, and aligned, resulting in a congruent sequence made up  
447 of contigs from both the forward and reverse sequences. The plant species were then identified by  
448 aligning the processed sequences against the GenBank database using the NCBI BLAST1 search  
449 engine. Subsequent phylogenetic analyses was done using the MEGA software version 11 (72).

450

## 451 **Metabolite extraction and analysis**

452 Metabolite compounds from cattle, camel, sheep, and goat rumen contents were extracted using  
453 the headspace, solid Phase microextraction (HS-SPME) technique as detailed by (73). Stableflex  
454 24Ga, manual holder SPME fibers (65 $\mu$ m, Polydimethyl Siloxane/Divinylbenzene (PDMS/DVB),  
455 Supelco, Bellefonte, Pennsylvania, USA) were used to trap the volatile metabolite compounds,  
456 and later analyzed by Gas chromatography (GC, HP-7890A, Agilent technologies, USA) coupled  
457 with Mass Spectrometry (MS, 5975C, Agilent technologies, USA), after which the compounds  
458 were identified as described by (73).

459

## 460 **Data analysis**

461 Multivariate statistical analyses were conducted based on the nature of the obtained data using R  
462 studio statistical software version 4.2.1 (74), PAST software Version 4.03 (75) and GraphPad  
463 Prism version 9. Similarity percentages (SIMPER) and One-way ANOSIM with Bray-Curtis  
464 dissimilarity index was used compare the profiles, and establish dissimilarity contribution of  
465 identified metabolite compounds based on their peak areas across the four livestock species. The  
466 metabolite compounds were then classified using the R software package “Random Forest”,  
467 version 4.2.1. The random forest analysis was executed by running 1000 iterations (ntree) with 10  
468 compounds randomly selected at each split ( $mtry = \sqrt{q}$ , where q is the total number of compounds.  
469 Based on the function ‘importance ()’ we generated the mean decrease in accuracy (MDA), which

470 provides an importance score for each metabolite compound. For each livestock, the metabolite  
471 with the highest MDA value was considered the most important. A multidimensional scaling plot  
472 (MDS) and a classical cluster dendrogram were used to visualize the output of analyzed metabolite  
473 compound profiles in each livestock. We then used Pearson's correlation to establish how  
474 metabolite compounds compared among individual ruminants' herd population. The detected  
475 metabolite compounds from across the 4 ruminants, were then pooled based on their chemical  
476 identities, after checking for normality using Shapiro-Wilk test ( $P > 0.05$ ), Pairwise comparison  
477 of the mean relative abundance of respective metabolite compounds in each chemical entity was  
478 analyzed by analysis of variance (ANOVA) among the four ruminants. Statistical significance was  
479 declared at  $P < 0.05$ .

480

#### 481 **Bioinformatics analysis**

482 Initially, the data obtained from Illumina sequencing was assessed using [nf-core-ampliseq](#) (v2.4.0)  
483 workflow and nextflow (v22.10.0), with predefined parameters of `truncLenf = 180` and `truncLenr =`  
484 `120`. The workflow proceeded as follows: first the quality of the reads were checked, using  
485 FASTQC (version 0.11.9). Cut adapt (v4.1) was then employed to trim reads and eliminate adapter  
486 sequences, following the method developed by Marcel Martin (76) . Preprocessing was performed  
487 using the DADA2 tool (v1.26.0) for filtering and trimming, dereplication, sample inference,  
488 merging of paired end reads, removal of chimeras, and taxonomic classification of the ASVs, as  
489 outlined by Callahan et al. (2016). Furthermore, DADA2 performed the classification of the ASVs'  
490 taxa based on their taxonomic categorization (Silva database v138 was used on 16S rRNA, and  
491 unite database v8.3 was used on ITS1 rRNA). Finally, Barnap tool (v0.9) was employed to predict  
492 the location of ribosomal RNA genes in genomes.

493

#### 494 **Abundance Visualization**

495 To visualize the ASV count table and ASV taxonomy table generated by the DADA2 algorithm  
496 within the nf-core ampliseq workflow, R statistical software (version 4.2.1) was used for further  
497 analysis. The ASV count table, ASV taxonomy table, and metadata were into a single phyloseq  
498 object using the Phyloseq package (version 1.40.0) in R. A `subset_taxa()` function was then

499 employed to eliminate undesired taxa before converting it to a data frame for further manipulation  
500 using the *phyloseq\_to\_df()* function. Subsequent data frame manipulation was conducted by  
501 tidyverse package (version 1.3.2). Lastly, ggplot2 (version 3.4.0) and Cairo (version 1.6.0) were  
502 used to produce the visual plots.

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513

514 **AUTHOR CONTRIBUTION**

515 VOO, designed, collected data, analyzed data, wrote the manuscript, MNG conceptualized,  
516 designed, analyzed data, wrote the manuscript, resource mobilization. CK, SM, and NVO  
517 contributed in the bioinformatics data analysis part of the work. GBO and JMO supervised,  
518 reviewed and edited the manuscript.

519 The authors declare no competing interests.

520

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522

523 **DATA AVAILABILITY STATEMENT**

524 The datasets generated during and/or analyzed during this study are all included in the manuscript  
525 and as supplementary materials.

526 **Supplemental Material**

527	<b>Table S 1</b>	<b>Bacteria population abundance</b>
528	<b>Table S 2</b>	<b>Fungi population abundance</b>
529	<b>Table S 3</b>	<b>List of identified metabolite compounds</b>
530	<b>Table S 4</b>	<b>Primer list for diet screening</b>
531		

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