Microbiome engineers:

Grazers, browsers, and the phytobiome stampede

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15   **Abstract**

16   We define *microbiome engineers* as species that modify the microbiome associated with other

17   host species via changes in the physical environment, potentially including the creation of

18   dispersal networks for microbiome consortia across multiple hosts. Grazers such as bison are

19   indirect plant microbiome engineers through alteration of the structure of plant communities in

20   grasslands and forests. They also can directly engineer plant microbiomes if they distribute

21   microbial consortia. Direct engineering may include simpler examples such as the role of

22   truffle-eating animals in structuring forest mycorrhizal communities, as well as more complex

23   roles in structuring the evolution of bacterial, fungal, and viral microbiome networks. Grazers

24   and browsers may have important historic and current roles in engineering microbiomes by (a)

25   stabilizing and homogenizing microbiomes in their preferred plant species, and (b) selecting for

26   microbiomes in their preferred plant species that are differentiated from microbiomes in plant

27   species they rarely consume.

28

29   Keywords: bison, browsing, community assembly, ecosystem engineers, grasslands, grazing,

30   microbiome, pathogens, phytobiome

31 Ecosystem engineers are species that manipulate the physical environment, apart from trophic

32 interactions, in ways that may affect numerous other species (Jones *et al.*, 1997). Beavers are a

33 classic example of an ecosystem engineer, constructing dams that support an ecological

34 community dependent on the resulting wetlands (Wright *et al.*, 2002) and connectivity among

35 wetlands (Hood and Larson, 2015). An individual plant may be considered an ecosystem

36 engineer through effects on the environment, including production of root exudates (Peiffer *et*

37 *al.*, 2013). We define a *microbiome engineer* as a species that directly and/or indirectly modifies

38 the spatial and temporal structure of the microbiome associated with other host species through

39 changes in the physical environment, potentially including creation of networks for dispersal of

40 microbial consortia across multiple host individuals (Fig. 1). This definition includes effects that

41 remove limitations to dispersal, in addition to effects on availability of resources (such as

42 nutrients) emphasized in definitions of ecosystem engineers. In contrast to more specific

43 vectors, a microbiome engineer influences larger microbial consortia. Humans may be, or may

44 become, the most influential microbiome engineer, developing synthetic communities and even

45 synthetic life forms (Mueller and Sachs, 2015).

46 The goal of this paper is to develop the concept of the microbiome engineer, particularly

47 in the context of vertebrate plant grazers and browsers, to illustrate the key role such animals

48 may play, and how high-throughput sequencing provides a new platform for understanding

49 engineering of whole consortia rather than simply a few taxa with known ecosystem roles.

50 Some links between mammals and plant-associated microbes are well-documented

51 (Johnson, 1996), such as how flying squirrels consume and spread ectomycorrhizal fungi and

52 nitrogen-fixing bacteria, structuring forest communities (e.g., (Li *et al.*, 1986; Lehmkuhl *et al.*,

53 2004)). Invasive mammals may facilitate the invasion of fungi and trees (Wood *et al.*, 2015).

54   Grazers and browsers may likewise spread many microbes through feces (Supplemental

55   Materials), but the process of grazing and browsing has particular potential for direct

56   microbiome engineering because it can introduce microbial consortia directly to wounded plant

57   organs that are adapted to recover. Transfer through grazing and browsing may also provide

58   protection against desiccation via saliva, and nutrients, for the microbial consortia introduced

59   into plant wounds. Microbes moved by microbiome engineers are co-evolved consortia, so there

60   may be a high probability of successful establishment. High-throughput sequencing opens the

61   door to studies of the movement of large assemblages of microbial taxa.

62   The American bison is well-known for its direct effects on plant communities (Hartnett *et

63   al.*, 1996; Knapp *et al.*, 1999; Towne *et al.*, 2005; Borer *et al.*, 2009), thus must have

64   corresponding important *indirect* effects on plant microbiomes, and may have *direct* effects by

65   moving microbes when grazing (Fig. 1). Cow saliva can disperse the fungus *Ascochyta paspali*

66   in *Paspalum dilatatum,* causing disease (Williams and Price, 1989). Bison have the potential to

67   act as direct microbiome engineers of prairies, because they maintain multiple microbial species

68   in their saliva, and thus may redistribute these microbial consortia through grazing (new data in

69   Supplemental Material). Grazing and browsing species may act as a hub or bridge node in

70   networks of microbial taxon association, where key microbial consortia associated with these

71   animals have a cascade of effects on other taxa (Faust and Raes, 2012; Poudel *et al.*, 2016).

72   Some functions of the taxa we recovered from bison saliva are known. *Fusarium* spp.

73   can produce mycotoxins, where the effects of mycotoxins in natural systems are mostly

74   unknown. In general, monogastric animals are much more sensitive to mycotoxins than are

75   ruminants such as bison. Species recovered from bison saliva play a role in regulating plant

76   growth (Harman *et al.*, 2004), and endophytic strains of *Fusarium* capable of synthesizing

77   gibberellic acid have previously been recovered from tallgrass prairie (Leslie *et al.*, 2004). An

78   isolate of the plant pathogen *Macrophomina phaseolina* from bison saliva was genetically

79   similar to an isolate from tallgrass prairie plants (Saleh *et al.*, 2010). New roles for microbial

80   species will continue to be discovered, *e.g.*, heat tolerance conferred by fungi infected with a

81   virus (Márquez *et al.*, 2007). Our data (Supplemental Material) focused on fungi, but grazers

82   such as bison also could disperse plant-associated viruses, bacteria, and nematodes.

83   Grazers and browsers may be important agents in shaping the spatial and temporal

84   distribution of plant-associated microbes. The combination of foraging decisions by grazers

85   such as bison (Courant and Fortin, 2010), and the pattern of grassland plant species co-

86   occurrence (Cox *et al.*, 2013), will help to determine the likelihood that particular plant species

87   are grazed in close enough temporal proximity to help homogenize their microbiomes. The

88   importance of microbiome engineers is determined by a number of such factors (Fig. 2),

89   including whether dispersal of microbes would otherwise be a limiting factor for community

90   assembly (Martiny *et al.*, 2006). If microbes dispersed by grazers and browsers produce

91   secondary metabolites in plants that alter the palatability of a plant, this feedback may produce

92   patterns that are self-reinforcing. Differences in grazing patterns and oral microbiome selection

93   by wild and domestic grazers and browsers could produce complex, overlapping microbiome

94   engineering effects.

95   A wide range of global vertebrate and invertebrate herbivores may be direct plant

96   microbiome engineers, including animals such as rabbits, giraffes, marsupials, geese, and

97   possibly even aquatic grazers such as sea turtles and manatees. One key feature of ecosystem

98   engineers is that their direct effects can outlive an individual engineer (Hastings *et al.*, 2007).

99   Microbiome engineers may influence the evolutionary trajectories of plant microbiomes long

100   after engineers have migrated to other areas (Odling-Smee *et al.*, 2013), and may re-introduce

101   taxa that are periodically or patchily less-favored, a process particularly important in annual

102   grasslands. The ability of bison saliva to maintain viable plant-associated microbes suggests a

103   potential role in long-distance dispersal and phytobiome engineering for other migratory

104   herbivores, *e.g.*, wildebeest and Cape buffalo in Africa, caribou and musk ox in the Arctic, and

105   kangaroos in the Australian Outback. Large mammal migrations may have engineered the

106   geographic structure of the plant microbiome across many of the world's grasslands, savannahs,

107   and forests.

108

109   **Conflict of Interest**

110   The authors declare no conflict of interest.

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112   **Acknowledgements**

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188   **Figure 1** A. Microbiome engineers as defined here affect microbial consortia, rather than only

189   individual microbial taxa (although a specialist vector could have indirect effects on the

190   phytobiome by modifying host health). B. Grazers and browsers are particularly well-positioned

191   to be microbiome engineers for living plant populations and communities because they create an

192   entry point to plants without killing plants. Many grazed/browsed plants are well-adapted to

193   recover and may regrow with the modified phytobiome. The mechanisms of grazer/browser

194   effects on plant microbiomes may be direct or indirect, via effects on plants. C.

195   Grazers/browsers may engineer plant microbiomes across individuals within a plant species, and

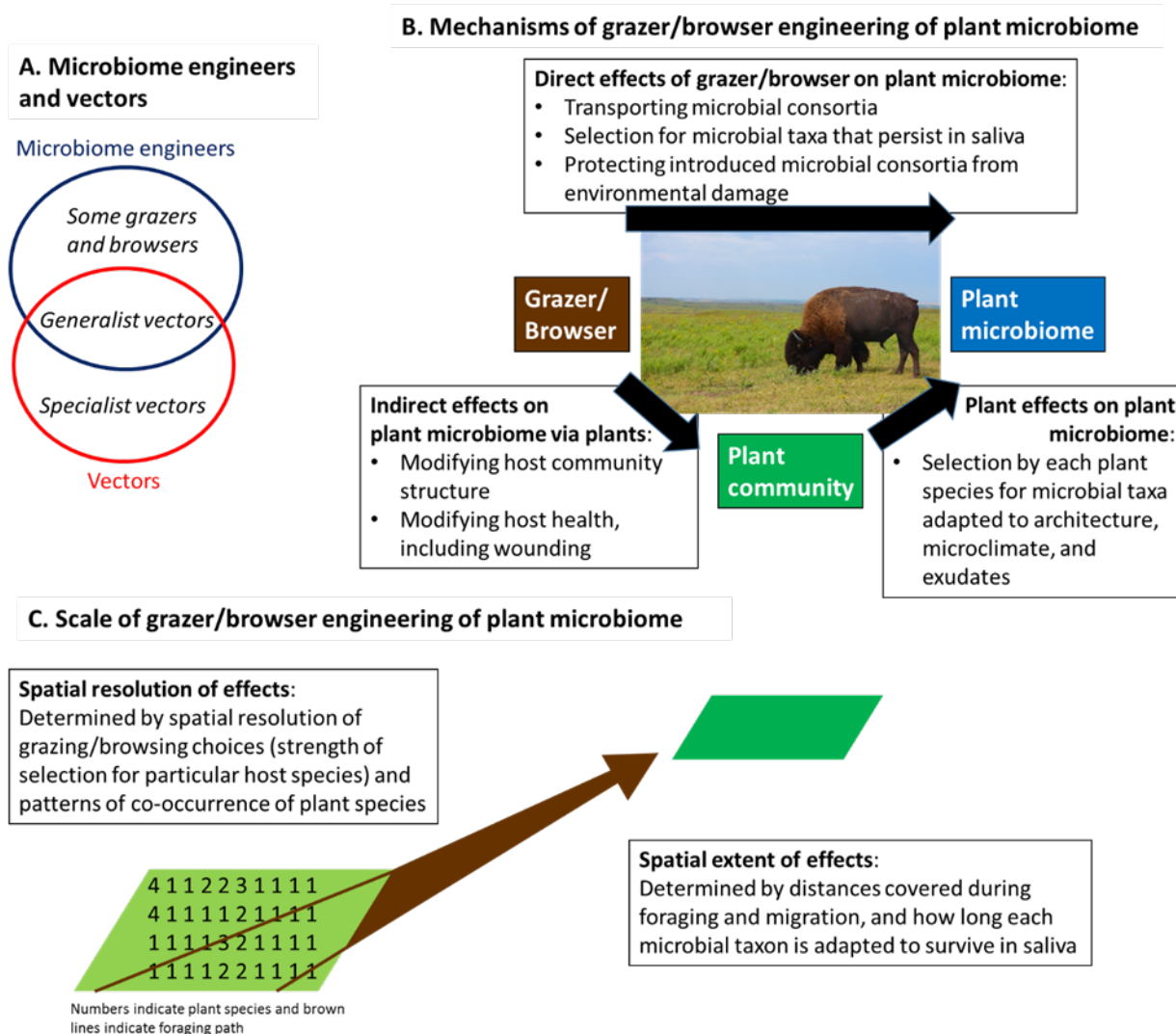196   across plant species. (Photo of bison by B. Van Slyke)

B. Mechanisms of grazer/browser engineering of plant microbiome

A. Microbiome engineers and vectors

Microbiome engineers

Some grazers and browsers

Generalist vectors

Specialist vectors

Vectors

Direct effects of grazer/browser on plant microbiome:
- Transporting microbial consortia
- Selection for microbial taxa that persist in saliva
- Protecting introduced microbial consortia from environmental damage

Grazer/Browser

Plant microbiome

Indirect effects on plant microbiome via plants:
- Modifying host community structure
- Modifying host health, including wounding

Plant community

Plant effects on plant microbiome:
- Selection by each plant species for microbial taxa adapted to architecture, microclimate, and exudates

C. Scale of grazer/browser engineering of plant microbiome

Spatial resolution of effects:
Determined by spatial resolution of grazing/browsing choices (strength of selection for particular host species) and patterns of co-occurrence of plant species

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4 1 1 2 2 3 1 1 1 1
4 1 1 1 1 2 1 1 1 1
1 1 1 1 3 2 1 1 1 1
1 1 1 1 2 2 1 1 1 1
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Numbers indicate plant species and brown lines indicate foraging path

Spatial extent of effects:
Determined by distances covered during foraging and migration, and how long each microbial taxon is adapted to survive in saliva
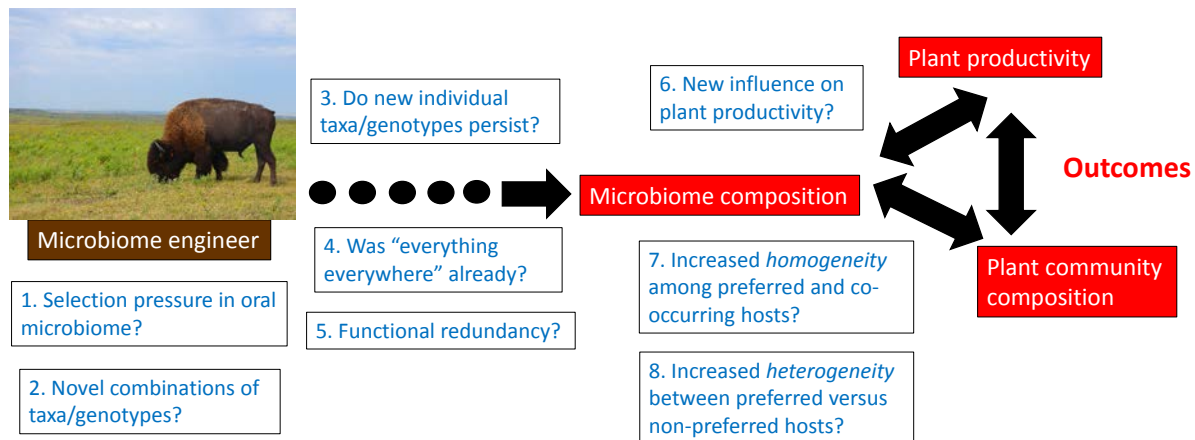
197

198   **Figure 2** Potential outcomes of direct microbiome engineering, and scenarios producing more or

199   less important effects

200   1. Does selection pressure in the oral microbiome, e.g., due to abundance and properties of

201   saliva, change the microbial consortium?

202   2. Does dispersal of microbial consortia produce new communities of taxa/genotypes that

203   would not assemble in plant microbiomes without grazing/browsing?

204   3. Do new individual taxa/genotypes introduced to the phytobiome persist?

205   4. Is it that "everything is everywhere" already, or does dispersal of microbial consortia

206   (especially into plant wounds) enable plant microbiome assembly?

207   5. Are new taxa/genotypes introduced to the plant microbiome functionally redundant with pre-

208   existing microbiome?

209   6. Do new taxa/genotypes or new combinations of taxa/genotypes influence plant productivity?

210   7. Does engineering result in increased microbiome homogeneity among individuals in

211   preferred plant species, and among plant species that tend to co-occur with them?

212   8. Does engineering result in increased microbiome heterogeneity among preferred versus non-

213   preferred plant species?

214   (Photo of bison by B. Van Slyke)

Some factors determining the importance of microbiome engineer effects

215

216 # Supplemental Material: Bison oral microbiome

217 **Microbiome engineers: Grazers, browsers, and the phytobiome stampede**

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224

225 **Background Information: Bison and Tallgrass Prairie**

226   Bison are known to have direct effects on the structure of tallgrass prairie plant

227 communities (Hartnett *et al.*, 1996; Knapp *et al.*, 1999; Towne *et al.*, 2005). In studies at Konza

228 Prairie Biological Station (KPBS, Manhattan, Kansas, USA), bison select grazing areas based on

229 four characteristics: a high population of the dominant tallgrass prairie plant species, lower forb

230 richness and diversity, lower plant species diversity, and high grass to forb ratios (Vinton, 1993).

231 Within bison grazing lawns, bison prefer particular plant species and areas in the lawns with

232 regrowth in grazed patches of high quality (Weaver and Tomanek, 1951; McNaughton, 1984).

233 The composition of bison diets changes as availability changes with the seasons. In early spring

234 and fall they spend more time in areas that are dominated by C_3 plants, while in the summer most

235 of their time is spent in areas dominated by C_4 grasses (Vinton *et al.*, 1993). Grazers may

236 indirectly support the spread of plant pathogens if they increase the abundance of highly-

237 competent hosts (Borer *et al.*, 2009). If they also spread plant-associated microbes, their grazing

238 patterns may help to structure the geographic patterns of plant microbiomes across time.

239   Plant-associated microbes are dispersed by numerous taxa, with the most widely

240   documented vectors being arthropods. Arthropods vector pathogens including bacteria, viruses,

241   fungi, and phytoplasmas. Other vectors include fungi, nematodes, and earthworms, while

242   vertebrates are less well-known as vectors of plant-associated microbes. Cow saliva can disperse

243   the fungus *Ascochyta paspali*, with inoculation of healthy plants by saliva containing fungal

244   conidia resulting in leaf blight disease in *Paspalum dilatatum* plants (Williams and Price, 1989).

245   Saliva effects on plants also have been evaluated in sheep, where saliva had positive effects on

246   plant growth (Teng *et al.*, 2010). Native grazers such as bison may play a major and

247   underappreciated role in the distribution of plant-associated microbes in grasslands.

248   The effects of bison grazing on plants have been evaluated in terms of the chemical

249   composition of bison saliva, and whether their saliva contains plant growth regulators (Detling *et*

250   *al.*, 1980; Detling *et al.*, 1981). Compensatory plant growth may occur in response to herbivory

251   (McNaughton, 1983). Some of the effects of saliva observed in the past may be due to microbes

252   contained in the saliva. Microbes in the saliva of grazers could be effectively inoculated into a

253   plant through grazing, as some saliva would remain on the plant wounds created by grazing and

254   might protect microbes from desiccation, and might also supply some nutrients. Microbes within

255   the saliva would be well placed to enter through the wounds and bypass many of the barriers

256   found in intact plant leaves and stems.

257   Plant-associated microbes play multiple roles in tallgrass prairies. Mycorrhizal

258   associations are important for the success of the dominant grass species (Hetrick *et al.*, 1988;

259   Hartnett and Wilson, 1999), and other fungi may positively influence plant growth (Harman *et*

260   *al.*, 2004). The role of plant pathogens in tallgrass prairie is less understood. Many pathogens

261   occur in tallgrass prairie (Tiffany *et al.*, 1990) with their prevalence both influenced by and

262   influencing plant community composition (Mitchell *et al.*, 2002; Barnes *et al.*, 2005). Fungicide

263   application can increase tallgrass prairie grass productivity and yields, suggesting the potential

264   importance of pathogens in limiting grass productivity (Mitchell, 2003; Dickson and Mitchell,

265   2010). New grassland fungus species, *e.g.*, *Fusarium konzum* (Zeller *et al.*, 2003), are likely to

266   be described as more attention is given to microbes in natural systems.

267   We sampled bison saliva for viable fungal propagules, to demonstrate the potential for

268   bison to spread microbial consortia while grazing.

269   **Methods in Bison Saliva Analysis**

270   *Sampling*. A herd of bison maintained at Konza Prairie Biological Station (KPBS)

271   (Towne, 1999) grazes native tallgrass prairie. Each autumn the bison are assembled for

272   weighing, offering an opportunity to sample their saliva. While briefly confined in the corral

273   during roundup, the bison had access to dried native prairie hay from KPBS that had been baled

274   in the summer. Thus, it is possible that the animals' access to the native prairie hay biased our

275   sample toward fungal species that can survive in dried prairie plant material. (During winter,

276   bison naturally feed on plant material dried in the field.) We sampled saliva from 51 bison (33

277   females and 18 males) with a single sterile veterinary swab per animal as individual animals

278   were held in a chute.

279   *Isolation of Viable Fungi.* Cotton swabs were streaked onto peptone/PCNB medium

280   (Leslie and Summerell, 2006). This medium is semi-selective for *Fusarium* spp. and other fungi

281   that can tolerate PCNB, streptomycin, and neomycin, and utilize an amino acid mixture as the

282   sole source of carbon and nitrogen. Swabs were then rotated 180° and streaked onto

283   *Macrophomina* selective media (Mihail, 1992). Plates were incubated at room temperature

284   (25±2°C) for 7-10 days. The extra step of culturing before sequencing makes it possible to say

285   definitively that the fungi were viable, whereas direct sequencing of DNA from the saliva also

286   would detect DNA from dead microbes.

287   Strains of interest were mass-transferred to Potato-Dextrose agar (PDA, Difco, Detroit, MI,

288   USA) slants and stored at 4°C for further purification and identification. For purification, strains

289   were transferred from PDA slants to Petri dishes containing peptone/PCNB medium or PDA

290   plates. Plates were incubated at 22°C with a 12 hr:12 hr light:dark cycle for up to two weeks.

291   Spores from colonies growing on the peptone/PCNB medium were streaked on a water agar slab

292   for microscopic examination and for subculturing single conidia to ensure culture purity. Three to

293   four spores were separated from each streak by micromanipulation. Slabs were incubated

294   overnight at 25°C. Germinated single spores were cut out of the agar slab under dissecting

295   microscope (50×) and transferred to Petri dishes containing Czapek's complete medium (Leslie

296   and Summerell, 2006). Cultures were incubated at 22°C with a 12 hr:12 hr light:dark cycle.

297   For morphological identification, pure colonies were subcultured to appropriate media and

298   fungal strains were identified according to Leslie and Summerell (2006) and Barnett and Hunter

299   (1998). DNA was extracted from cultures that had been grown on liquid complete medium and

300   incubated on an orbital shaker (150 rpm) at room temperature for 48-72 hours by using a

301   cetyltrimethylammonium bromide (CTAB) procedure (Leslie and Summerell, 2006).

302   *Sequencing.* Two genes were amplified and sequenced to help identify strains to species

303   level: β-tubulin (*tub-2*) and the internal transcribed spacer (ITS1 and ITS2) region of nuclear

304   rDNA gene (ITS-rDNA) (White *et al.*, 1990; O'Donnell *et al.*, 2000; Saleh *et al.*, 2010). The

305   DNA sequences were edited and aligned with BioEdit software

306   (http://www.mbio.ncsu.edu/Bioedit/bioedit.html). Cleaned DNA sequences were searched

307   against the GenBank database to identify identical or closely related fungi to assign strains to a

308   genus and species wherever possible.

Results and Discussion of Bison Saliva Analysis

310   A range of fungal taxa were recovered, with 30 fungal cultures identified to species level

311   (Table S1). Among the dematiaceous fungi recovered from bison saliva, one isolate was

312   identified as *Macrophomina phaseolina* and had the same haplotype as the MPKS327 strain

313   previously recovered from *Helianthus* sp. growing in a Kansas tallgrass prairie (Saleh *et al.*,

314   2010). The fungal species most frequently recovered (15/65 isolates) was *Phoma sorghina*,

315   which can cause foliar diseases in grasses and other plants. Among the hyaline-hyphae fungi,

316   isolates from three *Fusarium* species complexes were recovered: *Fusarium equiseti*, *Fusarium*

317   *oxysporum*, and *Fusarium solani*. Members of these *Fusarium* species complexes are all

318   ubiquitous soil inhabitants. Their presence may be due to an endophytic or pathogenic plant

319   association, or they may be non-pathogens present temporarily or growing as saprophytes on

320   plant surfaces. While some of the recovered species are well-known plant pathogens, such as *M.*

321   *phaseolina*, *Stagonosporopsis cucurbitacearum* and *P. sorghina*, others may be pathogens,

322   symbionts or hitchhikers, with their role dependent on the plant host and environmental

323   conditions (Newton *et al.*, 2010).

324   Viable fungi were recovered from 41/51 bison on peptone/PCNB medium, and from

325   14/51 bison on *Macrophomina* selective medium. There were 65 cultures for evaluation

326   following the single-spore subculturing process. Based on morphological characterization, the

327   recovered fungal strains were divided into two groups: fungi with hyaline hyphae and fungi with

328   dark-colored hyphae (dematiaceous fungi). We amplified and sequenced both the *tub-2* and ITS-

329   rDNA regions from 43/65 fungal strains. For the remaining 22 strains either the ITS-rDNA (17

330   strains) or *tub-2* (5 strains) region was amplified and sequenced. When these DNA sequences

331   were searched against the GenBank database, 57/65 fungal cultures were identified to the genus

332   level with an average 99% identity for ITS-rDNA regions and 90% identity for *tub-2* regions. In

333   addition, 30 out of the 57 fungal cultures were identified to the species level (Tables S1).

334   Among the hyaline-hyphae fungi, members of three Fusarium species complexes were

335   recovered: *F. equiseti* (seven strains with ITS-rDNA and tub-2 sequences 100% and 95%

336   identical to sequences already deposited in GenBank, respectively), *F. oxysporum* (four strains

337   with sequences 100% identical to those deposited in GenBank for both sequenced regions), and

338   *F. solani* (one strain with sequences 99% identical to those deposited in GenBank for both

339   regions). Fungal strains identified as *Plectosphaerella* sp. (5 strains), *Verticillium* sp. (4 strains),

340   *Aspergillus tubingensis* (1 strain), *Acremonium* sp. (1 strain), and *Colletotrichum* sp. (1 strain)

341   also were recovered. While identification to genus is not sufficient to evaluate the ecological

342   role for these taxa, all of them could be plant pathogens. There were eight strains with less than

343   90% DNA sequence similarity to any of the DNA sequences available in GenBank.

344   Recovery of multiple species of viable fungi indicates the potential for bison to directly

345   engineer grassland microbiomes, in addition to their indirect effects on the microbiome through

346   structuring plant communities. The common impression that soilborne and endophytic fungi are

347   limited to very gradual dispersal, through soil movement and plant growth, must be modified to

348   take into account dispersal by bison and probably other grazers. Bison have historically been

349   one of the most abundant large land mammals known, with perhaps 50 million bison ranging

350   from Canada to Mexico around 2000 years ago. Bison travel approximately 3 km a day in

351   typical grazing patterns, but have historically moved much greater distances in seasonal

352   migrations (Meagher, 1986). The range of the ~ 10,000 free-ranging animals today (Meagher,

353   1986; Nowak, 1999; Lott, 2002) is much more limited and does not include the vast majority of

354   the Great Plains region where they roamed freely until 100-150 years ago, dispersing microbes

355   as they grazed.

356   Bison have the potential to directly engineer both plant microbiomes and soil

357   microbiomes more broadly. Soilborne microbes could be moved over the period of several days

358   or longer, including movement in bison fur (from dust wallows) and hooves, and carried a

359   considerable distance, especially to other wallows. Plant-associated microbes could be moved in

360   saliva across distances covered in one to a few days, or potentially longer, while they remained

361   viable in an animal's mouth. If the microbes were swallowed, those that survive the animal's

362   digestive system could be dispersed in the dung. Members of all three of the *Fusarium* species

363   complexes recovered, for example, produce thick-walled chlamydospores that could transit the

364   bison's digestive tract. 96% of the DNA sequences identified from the dung of pronghorn

365   antelope (*Antilocapra americana*), which also graze the Great Plains, were categorized as *Phoma*

366   species (Herrera *et al.*, 2011). The predominance of viable *Phoma* propagules among the fungi

367   we recovered from bison saliva could indicate that this important group of plant pathogens has

368   evolved in concert with large herbivores in the Great Plains, helping to insure dispersal

369   throughout the region. The profile of bacteria observed in bison dung suggests differences that

370   may reflect bison health status (Weese *et al.*, 2014), and bison health status might help determine

371   which microbes survive in saliva or dung.

372   Unlike microbe transport in soil or deposited in dung, bison saliva is ideally positioned

373   for plant infection. As the animals graze, they create wounds to which saliva would be

374   simultaneously applied. Organisms transmitted in saliva thus need fewer long-term survival

375   structures and lesser abilities to abrogate or bypass plant defenses because they would effectively

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376   be injected directly into the plant vascular system. These microorganisms could have adapted to

377   short-term and possibly long-term survival in the bison oral microbiome in much the same way

378   that other microorganisms have adapted to survive in much better understood arthropod vectors.

379   Global grassland microbiomes likely retain some spatial structure based on historic

380   grazing patterns, while more recent replacements of wild grazers with domesticate animals may

381   continue to modify these patterns.

382

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514   Table S1. Viable fungi recovered from bison saliva, identified using morphological and

515   molecular markers

Number of isolates	Fungal taxon
15	*Phoma sorghina*
4	*Fusarium oxysporum*
2	*Stagonosporopsis cucurbitacearum*
1	*Aspergillus tubingensis*
1	*Fusarium solani*
1	*Macrophomina phaseolina*
15	*Phoma* species complex
7	*Fusarium equiseti* species complex
5	*Plectosphaerella* sp.
4	*Verticillium* sp.
1	*Acremonium* sp.
1	*Colletotrichum* sp.
8	Ascomycete

516