The Unicorn Microbiome: A proposed microbial mechanism for the rainbow pigments in unicorn poop.

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Summary:

Unicorns (Equus unicornis) are a rare terrestrial mammal, which have been noted to produce a variegated pigmented feces that is often referred to as "rainbow poop." Despite considerable commercial interest in unicorn rainbow poop, the mechanism leading to this rainbow-pigmentation remains unknown. Microbial metabolismincluding the production of pigments- can influence the perceived color of animal excreta. To determine if the rainbow pigments of unicorn feces could have a microbial origin, we used a bioinformatic and microbial cultivation-based approach. To establish what microorganisms could be producing such colored pigments, we used a comparative in silico approach to characterize the unicorn microbiome. As microbiomes often have host phylogenetic signatures, we indirectly measured the unicorn microbiome by determining those bacterial taxa found in the published microbiomes of unicorn nearest extant sister species: the horse and narwhal. For those bacterial phyla which were shared by horses and narwhals, and thus likely part of the core unicorn microbiome, we selected representative cultivated species to establish pigment production capability. To elucidate what pigments were predictably in unicorn feces we used a greedy search algorithm scanning a library of unicorn feces images uploaded by citizen scientists (aka: Google Image Search). We then compared pigments produced by these representative microbes to those pigments found in unicorn feces. Our model predicted the unicorn microbiome to consist of Actinobacteria, Bacteroides, Firmicutes, Proteobacteria, and a subset of additional taxa. Representative strains from these major phyla (e.g. Serratia marcescens, Methylobacteria sp., Rhodococcus hoagie, Micrococcus luteus, Streptomyces coelicolor, Janthinobacterium lividum, and Cyanobacteria species) produced pigments with the following colors: red, orange, yellow, green, blue, purple, and pink. These colors represent the pigments observed in the majority of observations of unicorn feces (pigments in at least >50% of images analyzed). We therefore propose that microbial pigments originating from bacteria within the unicorn microbiome may be responsible for the characteristic rainbow patterns observed in unicorn feces. As microbial pigments are useful to various industries, the unicorn microbiome presents an opportunity for future targeted bioprospecting efforts.

Keywords: unicorn, poop, microbiome, variegated pigmented feces.

Introduction:

It is well established that a defining trait of unicorns (*Equus unicornis*) is their ability to produce feces with concentric bands of refracted and reflected visible light (rainbow colored poop). Despite the biological novelty of this trait and the considerable commercial interest in this animal byproduct (e.g. CNBC, 2018), the mechanism behind this phenomenon remains unknown.

Animal feces can be influenced by host-specific and environmental factors. For example, humans (*Homo sapiens*) produce red or green-pigmented feces after ingesting plant material such as beets, which contain pigmented beta-carotenes and chlorophyll (respectively). Such understandings have been leveraged to modify the diets of specific zoo animals to later assist with excreta identification for hormone metabolite studies (e.g. Fuller et al. 2010). Animal feces color may also be affected by contributions from host tissue, such as when the presence of blood in feces during disease states produces a characteristic dark brown appearance. This has led to the development of diagnostics for ulceration of the digestive tract (Aaron, 1924). Beyond diet and host metabolism, microorganisms—and their metabolic byproducts—are often the major contributor to fecal pigments.

The microbiome of animals—and consequently their feces— contains diverse microorganisms that assist with host nutrition and immune function (Lee and Hase 2014). These microorganisms produce diverse metabolites, many of which are pigments visible in the UV spectra (Rao et al. 2017). Singular species can be responsible for such pigments in animal excreta, such as when certain *Proteus mirabilis* infections in humans lead to purple urine bag syndrome (Peters et al. 2011). Communities of species can also impact fecal pigment, as in the case of human feces presenting as a brown color as a result of microbial community metabolism of bilirubin (Tiribelli and Ostrow, 2005).

Here, we investigated the microbiome of unicorns and elucidated the potential microbial origin of their characteristic rainbow-colored fecal pigments.

Methods:

Determination of rainbow colors in Unicorn feces:

We used the greedy search algorithm (PageRank) with an existing reference database of images contributed by citizen scientists (Google Image Search) to obtain a haphazard collection of representative images of unicorn feces (n=6) (Page et al. 1999). We specifically used the text string "Unicorn poop." Similar results were found for the additional text sting "unicorn feces," but only the "unicorn poop" results are further analyzed here. We then qualitatively assessed the pigments within the images of feces to determine a confidence score for what colors were most frequently observed in the average "rainbow" of unicorn feces.



Figure 1. Images used to determine what colors are most frequently found in unicorn poop.

Microbiome Assessment:

Sampling:

We faced challenges in obtaining sufficient samples of unicorn feces (n = 0) due to the paucity of available hosts in captivity, and the lack of reliable museum specimens. We also faced IACUC permit issues regarding the sampling of exceptionally rare species. We therefore performed an *in silico* comparative analysis to determine the expected core microbiome of unicorns (See descriptions of the core microbiome in Shade and Handelsman, 2011).

Animal microbiomes are often similar across phylogenetically related species (Groussin et al. 2017). Therefore, to indirectly determine the unicorn microbiome we compared the published microbiomes of unicorns' nearest phylogenetic neighbors: narwhals and horses (See Marielle and Case, 2012 for genomic evidence of hybridization).

Bioinformatics:

We identified the shared bacterial phyla of horses (Dougal et al. 2013) and narwhals (Sanders et al. 2015) and determined this to be the inclusive core microbiome of the hybrid: the unicorn microbiome.



Figure 2. Schematic for Unicorn microbiome model predictions.

Microbial pigment production:

To determine if microorganisms within the unicorn microbiome have the capacity to produce rainbow pigments, we cultivated representative species from the dominant unicorn microbiome lineages *in vitro* on nutrient media. Following incubation we qualitatively assessed color by comparing pigments to the RBG color scale. For those reference microorganisms we could not cultivate, we obtained published pictures for pigment analysis.

Results and Discussion:

Using our informatic approach, paired with citizen provided images, we determined that unicorn rainbow feces frequently consisted of the colors red, orange, yellow, green, blue, purple, and pink pigments (**Table 1**).

Percentage of fecal images featuring this color
66.7
66.7
100
100
100
66.7
50

Table 1. Heatmap of the colors found in unicorn feces images based on frequency of observed color across images analyzed.

The core microbiome of unicorns included bacterial phyla that were present in both horse microbiomes and narwhal (toothed whale) microbiomes. They included the phyla Actinobacteria, Bacteroides, Firmicutes, Proteobacteria, and a subset of additional taxa that were unclassified, or classified as "other" (**Table 2**).

Table 2. Bacterial phyla in various		
Bacterial Phyla in Horse Microbiome (from Dougal 2013)	Bacterial Phyla in Toothed Whales Microbiome (from Sanders et al 2015)	Predicted Phyla in Unicorn Core Microbiome(a)
Firmicutes	Proteobacteria	Proteobacteria
Bacteroidetes	Actinobacteria	Firmicutes
Proteobacteria (family)	Fusobacteria	Bacteroidetes
Fibrobacteres	Firmicutes	Actinobacteria
Unclassified (cyanobacteria)	Tenericutes	Other
Spirochaetes	Other	
Actinobacteria	Bacteroidetes	
Other		
a Predicted phyla based on phyla being present in both the horse and the toothed whale microbiomes		

We assessed the pigment production capability of representatives of these major phyla: Proteobacteria (*Serratia marcescens, Methylobacteria* sp.), Actinobacteria (*Rhodococcus hoagie, Micrococcus luteus, Streptomyces coelicolor, Janthinobacterium lividum*), Unclassified/other (Cyanobacteria). On various media, these microorganisms produced the observed colors red, orange, yellow, green, blue, purple, and pink (**Figure 3**). These colors represent the pigments observed in the majority of observations of unicorn feces (colors in at least >50% of images analyzed) (**Table 2**). These results suggest a putative microbial metabolic role in the production of rainbow pigments observed in unicorn feces.

A limitation of this study was that pigment production was assessed in vitro, but not within the feces. It is well-established that microorganisms produce different pigments in different environments and under different culture conditions (e.g. Chen and Johns, 1993); therefore, future *in vivo* studies are needed to understand how our results and model predictions compare with microbial pigment production of microbes within the unicorn GI tract.



Figure 3. Representative images of the following bacteria producing natural pigments (from left to right: *Serratia marcescens, Rhodococcus hoagie, Micrococcus luteus,* Cyanobacteria species, *Streptomyces coelicolor, Janthinobacterium lividum,* <u>*Methylobacterium*</u> sp.)

Microbial pigments are used across various industries. Some are useful for the pigment themselves, such as microbes that help produce indigo dyes for textiles (Park et al. 2012), pigments used in food production to enhance organoleptic attributes (Dufosse 2006), or those microbes and pigments used by bioartists and educators to create bacterial art (Charkoudian et al. 2010). Others are useful because of additional activities of the compounds unrelated to the their light reflectance and refraction. For example, *Streptomyces coelicolor* produces the blue pigment Actinorhodin, a compound with antibiotic activity against gram-positive bacteria (Wright and Hopwood, 1976). Our research suggests that the unicorn microbiome, and specifically unicorn feces, may be a previously untapped source for novel pigment-producing strains.

Conclusion:

We predict that the unicorn microbiome is composed of bacterial taxa from the Actinobacteria, Proteobacteria, Firmicutes, Bacteriodetes, and Cyanobacteria phyla. Representatives of these taxa produce pigments when cultivated, and these same pigments are those most frequently observed in images of unicorn feces. Such pigments include the colors red, orange, yellow, green, blue, purple, and pink. We therefore propose that the color of unicorn rainbow feces may be the result of spatially segregated microorganisms producing pigments. Ultimately, this information may benefit future bioprospecting efforts targeting the discovery of novel pigment-producing microbes.

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