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Artículo de investigación

The effect of clay supplementation on fecal consistency, parasites, and gut microbiome in captive macaques

El efecto de la suplementación con arcilla sobre la consistencia fecal, los parásitos y el microbioma intestinal de macacos en cautiverio

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Abstract

Geophagy, or the consumption of earth materials, is prevalent across primates, including humans. The main drivers of this behavior are unknown. However, often the consumed materials have high amounts of clay, which can absorb water, bind plant secondary compounds, supplement minerals, alter the gut pH, and influence the gut microbiota. As a result, clay supplementation could have a range of potential health applications. Yet, few studies have evaluated the potential benefits or risks of clay consumption. To begin to address this knowledge gap, we describe the effects daily clay supplementation for one week on fecal consistency, parasite burdens, and gut microbiome composition in 14 healthy captive macques (*Macaca* spp.). We trialed two food-grade clay minerals, kaolinite and montmorillonite, using several delivery modes. We found that the monkeys consumed the clay supplements and preferred kaolinite delivered in either peanut butter or bananas. The overall health of the monkeys was minimally affected by clay supplementation, both during the one-week supplementation trial period and for three weeks after. In response to clay supplementation, fecal consistency remained 'normal' for 67% of the monkeys; however, 33% developed "clay-like" feces, suggesting that the amount of clay received was too high. Parasite prevalence increased during the treatment phases, potentially due to more frequent sampling, but the composition of the microbiome was mostly unaffected. Clay is known to be a powerful binding agent, but these results suggest that it may not adsorb or affect the microbiome, which antibiotics may do. Overall, our results suggest that clay supplementation does not have short-term negative health effects in healthy captive macaques and should be tested in other primates and humans. We encourage longer trials with additional primate species to 1) determine a safe and effective dose that reduces negative side-effects like constipation, 2) to evaluate the effect of food vehicles, and 3) to determine the effect of clay on other systems and functions.

Keywords: soil eating, geophagy, nonhuman primates, kaolinite, montmorillonite.

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Resumen

La geofagia, o consumo de tierra, es común entre los primates, incluidos los humanos. Se desconocen los principales motivos de este comportamiento. Sin embargo, a menudo los materiales consumidos tienen altas cantidades de arcilla, que pueden absorber agua, ligarse a compuestos secundarios de plantas, complementar minerales, alterar el pH intestinal e influir en la microbiota intestinal. Como resultado, la suplementación con arcilla podría tener una variedad de posibles aplicaciones para la salud. Sin embargo, pocos estudios han evaluado los posibles beneficios o riesgos del consumo de arcilla. Para comenzar a abordar esta falta de conocimiento, describimos los efectos de la suplementación diaria con arcilla durante una semana sobre la consistencia fecal, la carga de parásitos y la composición del microbioma intestinal en 14 macacos cautivos sanos (*Macaca* spp.). Probamos dos minerales arcillosos aptos para alimentos, caolinita y montmorillonita, usando varios modos de entrega. Descubrimos que los monos consumían los suplementos de arcilla y preferían la caolinita entregada en mantequilla de maní o plátanos. La salud general de los monos se vio mínimamente afectada por la suplementación con arcilla, tanto durante el período de prueba de suplementación de una semana como durante las tres semanas posteriores. En respuesta a la suplementación con arcilla, la consistencia fecal permaneció "normal" para el 67 % de los monos; sin embargo, el 33 % desarrolló heces "parecidas a la arcilla", lo que sugiere que la cantidad de arcilla recibida fue demasiado alta. La prevalencia de parásitos aumentó durante las fases de tratamiento, posiblemente debido a un muestreo más frecuente, pero la composición del microbioma no se vio afectada en su mayoría. Se sabe que la arcilla es un poderoso agente aglutinante, pero estos resultados sugieren que es posible que no se absorba ni afecte al microbioma, lo que sí pueden hacer los antibióticos. En general, nuestros resultados sugieren que la suplementación con arcilla no tiene efectos negativos a corto plazo para la salud en macacos cautivos sanos y debe probarse en otros primates y humanos. Se sugiere llevar a cabo ensayos más prolongados con otras especies de primates para: 1) determinar una dosis segura y eficaz que reduzca los efectos secundarios negativos como el estreñimiento, 2) evaluar el efecto de los alimentos utilizados y 3) determinar el efecto de la arcilla en otros sistemas y funciones.

Palabras clave: comer tierra, geofagia, primates no humanos, caolinita, montmorillonita.

Introduction

Geophagy, or the deliberate consumption of earth materials, is prevalent in humans and non-human primates (Tateo & Summa, 2007; Young, 2012; Young & Miller, 2019; Pebsworth et al., 2019). Geophagy, referred to as pica in humans, has been observed on all inhabited continents. Archeological evidence suggests that soil eating dates to *Homo habilis* and continues today (Clark and Kleindienst, 2001). In 237 out of 243 cultural reports, people described the soil they eat and prefer as clay-like (Young et al., 2011). In the wild, 136 species of non-human primates (NHP) are known to deliberately consume earth materials rich in clay (Pebsworth et al., 2019). There are two non-mutually exclusive hypotheses for geophagy: protection of the gastrointestinal (GI) tract and supplementation of micronutrients. Clay has been used to improve GI health outcomes in some contexts, and it has a long, rich history in human therapeutic practices (Tateo & Summa, 2007; Young, 2012; Young & Miller, 2019). The clay minerals kaolinite and smectite are used in pharmaceutical preparations to control diarrhea, heartburn, nausea, and upset stomach (Das et al., 2015; Dupont & Vernisse, 2009; Vermeer & Ferrell, 1985). Clay minerals are effective treatments for diarrhea in suckling and weaned piglets (Bederska-Łojewska et al., 2016; Song et al., 2012; Vondruskova et al., 2010), and for 'heat' diarrhea in foals (Pieszka et al., 2016). They also reduce subacute ruminal acidosis in cattle (Humer et al., 2019). With an increase in microbial drug resistance, there has been a resurgence of interest in clay minerals' ability to protect the GI tract from bacteria and other pathogens (Khezerlou et al., 2018; Londono et al., 2017).

There are multiple mechanisms through which clay can positively influence GI health. First, clay functions as a detoxifying agent because of its sorptive properties, or ability to adhere to ions or molecules from another substrate (Rezvani & Taghizadeh, 2018). Clay can adsorb plant secondary compounds such as tannins and alkaloids that are produced to deter herbivores and reduce palatability, inhibit proteolytic enzymes, affect feeding rates, and even reduce reproductive success (DeGabriel et al., 2009; Mahandran et al., 2015; Pebsworth, Hillier, et al., 2019; Talukdar & Ghosh, 2018). Clay also detoxifies mycotoxins, fungal secondary metabolites found in human food and animal feed that can present significant health risks such as hepatocellular carcinoma (D'Ascanio et al., 2019; Dixon, et al., 2008; Gouda et al., 2019; Phillips et al., 2008; Phillips et al., 2019). Finally, clay affects the microbial communities in the GI tract. Clay nanoparticles inhibit a variety of parasites by disrupting the proliferation and metabolic activity of promastigotes, inactivating oocysts, impairing parasites' ability to infect, and/or damaging DNA (Khezerlou et al., 2018). Clay also adsorbs bacteria (e.g., *Pseudomonas aeruginosa*, *Escherichia coli*) known to cause severe diarrhea (Said et al., 1980; Vondruskova et al., 2010). Because differences in the relative abundances of bacterial taxa that are not obvious pathogens have also been associated with diarrhea and GI disease in several contexts (DuPont, 2009), it is possible that clay also functions by altering the overall gut bacterial community through adsorption or other mechanisms. For example, clay has been reported to reinforce the mucosal barrier and reduce intestinal inflammation (González et al., 2004). Although these effects could be a direct result of the clay itself, gut bacteria have been shown to affect the same aspects of host physiology (Hiippala et al., 2018) and could also mediate the effects of clay on GI symptoms.

Nevertheless, concerns regarding the potential risks of clay consumption remain. There is fear that eaten clay may harbor geohelminths, pathogenic bacteria, or heavy metals that could exacerbate consumer GI symptoms or trigger other negative health outcomes (Al-Rmalli et al., 2010; Geissler et al.,1998; Kutalek et al., 2010; Sumbele et al., 2014). Clay may also bind up nutrients and beneficial

pharmaceuticals (Young & Miller, 2019), and one study indicated that clay supplementation may cause oxidative stress, reduction in cell viability, apoptosis, and DNA damage in livestock (Elliott et al., 2019). Ingested clay may also cause dental and tissue abrasion and severe constipation (Hunter-Adams, 2016; Toker et al., 2009). Additionally, in human subjects, calcium montmorillonite supplementation caused bloating, constipation, or other mild GI complaints. However, supplementation did not cause any significant difference in hematology, kidney function, electrolytes, vitamin A and E, and minerals (Wang et al., 2005).

Importantly, our understanding of the effects of clay on consumer health is constrained to a narrow collection of animal species and contexts. As a result, it is currently difficult to systematically assess the benefits and risks of clay supplementation. Furthermore, knowledge of how clay interacts with specific parasites or pathogenic bacteria in different animal hosts remains relatively limited, and few studies have explored the effect of clay on the broader gut bacterial community (Neubauer et al., 2019).

To begin to address some of these knowledge gaps, we conducted a pilot study to assess the effect of clay supplementation on various aspects of GI health in a small sample of healthy captive macaque monkeys (*Macaca* spp.). Macaques are Old World monkeys--specifically belonging to the subfamily Cercopithicinae--commonly used as models for biomedical research in humans. Like most cercopithecines, macaques consume an omnivorous diet, making their digestive physiology similar to that of humans. Additionally, the gut microbiome of humans is more similar to that of cercopithecine primates than any other group of primates, including apes (Amato et al., 2019). Therefore, our results are relevant for understanding the potential health effects of geophagy on macaques as well as other omnivorous primates, including humans. Specifically, we aimed to 1) determine the extent to which macaques would consume two types of clay minerals, kaolinite and montmorillonite, and 2) assess the impact of clay minerals on fecal consistency, parasite burdens, and gut bacterial community composition. We predicted that consumed clay minerals would increase fecal consistency, decrease parasite burdens, and alter the gut bacterial composition. Because montmorillonite is a stronger sorptive agent than kaolinite, we predicted that it would have stronger effects.

Methods

Subjects and housing conditions

We conducted clay supplementation trials at Primarily Primates, Inc., a sanctuary in San Antonio, Texas, from October 19 to November 28, 2018. Because this is not a controlled breeding colony, we were not able to control for macaque species, age, sex, or reproductive status. Instead, our goal was to examine the short-term health impacts of clay supplementation on the range of healthy individuals available to us.

We used fourteen monkeys from the genus Macaca: four *M. fascicularis*, one *M. fuscata*, eight *M. mulatta*, one *M. nemestrina*, one *M. radiata*, and one *M. mulatta X M. fascicularis* hybrid (Table S1). Twelve males and two females participated. Their ages ranged from 4 – 30 years, with a mean age of 15.6 (SE) years. Six monkeys were previously used in research. Because retired research animals have their research protocols redacted from their medical record before they are released to sanctuaries, we did not have specific information about the research history of these individuals. Eight monkeys were surrendered pets. Reproductively, seven were intact, six vasectomized, and one was castrated.

Before the clay supplementation trial began, one monkey was experiencing idiopathic diarrhea that was not remedied with dietary supplements (e.g., probiotics, Benefiber®). However, fecal consistency returned to normal before this individual began to receive clay supplements. All dietary supplements and medications ceased for all individuals one month before the clay supplementation trial began. All monkeys received the same diet: 2% of body weight/day Mazuri ® Primate Basix monkey biscuits and 2% of body weight in produce. For example, monkeys were given one fruit (e.g., apples) and two different vegetables (e.g., corn, sweet potatoes) per day to achieve a 2/3 vegetable, 1/3 fruit ratio. We used the same produce from the same vendor for all monkeys that took part in the study. Water was available *ad libitum*.

All study subjects were single-housed. To ensure there was no microbial contamination from a natural substrate, we selected individuals that were housed in concrete-floored corncrib habitats. For the duration of the study, they remained in the same habitat. Daily, the staff cleaned the enclosures with a high-pressure hose. Weekly, they also disinfected the enclosures with bleach, and subsequently rinsed with water.

Clay supplementation

We offered each monkey clay minerals, 0.03% of the diet based on body weight. Throughout the manuscript, this will be referred to as clay "supplementation". Initially, seven test subjects were offered food-grade kaolinite and seven sodium montmorillonite. However, two monkeys that were initially offered montmorillonite refused to consume it and were shifted to the kaolinite treatment. As a result, nine test subjects consumed kaolinite and five sodium montmorillonite once daily for five days (25 – 29 October 2018).

The amount of clay offered daily ranged from 0.96 – 1.68 grams based on the weight of the test subject. To encourage the monkeys to eat the clay, we offered it in five different vehicles (favored foods): day 1, grape jelly; day 2, applesauce; day 3, peanut butter; day 4, peanut butter; and day 5, banana with honey. We monitored consumption and documented amount eaten (refused, ate some (<1-50%), ate most (> 50% but < 100), ate all). Because clay minerals can potentially adsorb micronutrients and drugs, as a precaution to avoid contact of clay and food in the stomach, we offered the clay at least two hours before food.

Animal welfare

Leading up to and during the study, all study subjects were visually evaluated at least thrice daily for well-being by their caregiver and/or the veterinarian. Housing standards for the subjects met welfare standards required by the USDA-APHIS (Act & Regulations,

2019). Dr. Elizabeth Pannill, the USDA Veterinary Medical Officer, reviewed and approved the protocol and procedures employed in this study and deemed them ethical. All produce offered during the study was inspected and approved as human table quality, cleaned, weighed, portioned, and prepared by the veterinarian.

Fecal collection

Fecal samples were collected at approximately 7-day intervals during five one-week periods: period 1= (10/19/18) pre-supplementation, period 2 = (10/25/18-10/29/18 supplementation, period 3 = (11/5/18-11/6/18) post-supplementation; period 4 = (11/16/18-11/17/18) post-supplementation; and period 5 = (11/26/18-11/27/18) post-supplementation. Feces were collected opportunistically within 2 hours after defecation using a plastic spoon that had been wiped with 95% ethanol. For each sample, staff reported consistency using a study-specific scale (1=firm, 2= normal, 3=soft). This scale was adapted from the Bristol stool scale. Because no subjects received a rating outside of the range of 2-3 on the Bristol scale, we adjusted the scale to describe more subtle variation within this normal range. They then stored aliquots sampled for parasite and gut microbiome analysis. For parasites, they mixed 1-2 grams of feces with 10% neutral buffered formalin. For gut microbiome analyses, they submerged the samples in 95% ethanol. Each vial was labeled with the name of the monkey and the date the sample was collected. We also recorded each monkey's age, sex, reproductive status, if antibiotics had been used within the last six months, and visible signs of illness (GI in particular). The same individual processed all fecal samples for delivery to VRL Laboratory and the Amato Laboratory.

Parasite analysis

Eleven monkeys tested positive one or more times during the past 26 months for *Entamoeba coli*, *Iodamoeba butschlii*, or both, so we focused on these two intestinal protozoans for this study. Prevalence data were generated commercially by VRL Laboratories, San Antonio, TX, a simian reference laboratory. They used a trichrome stain technique to identify both parasites.

Microbiome analysis

Samples were shipped to the Amato Lab at Northwestern University for analysis. DNA was extracted using the Qiagen DNEasy PowerSoil kit with modifications and a two-step polymerase chain reaction (PCR) was used to amplify the V4-5 region of the 16S rRNA gene using the 515f and 926r primers (Walters et al., 2016) as described previously (Mallott & Amato, 2018). Extraction and PCR negatives were used to control for contamination. PCR products were purified and normalized using a SequalPrep Normalization Plate and sequenced on the Illumina MiSeq V4 platform at the University of Illinois Chicago DNA Services Facility. Raw DNA sequences are available in the Sequence Read Archive (accession # available upon request).

Raw 16S rRNA gene amplicon sequences were trimmed, quality-filtered, and dereplicated, amplicon sequence variants (ASVs) were inferred, and paired reads were merged using the DADA2 algorithm (Callahan et al., 2016) within QIIME2 (v2019.7) (Bolyen et al., 2019). After these filtering steps, our dataset contained 766,759 reads with an average of 10,223 per sample (range 6,690 to 21,597). Taxonomy was assigned in QIIME2 using a Naive Bayes classifier trained on the Greengenes 13_8 99% OTU database using the full 16S rRNA gene sequence lengths. Mitochondria and chloroplast ASVs were filtered from the dataset. We generated alpha rarefaction curves using alpha-rarefaction, and based on the output, chose not to discard any samples since all had sufficient depth to exhibit a plateau in diversity.

We used the breakaway wrapper in QIIME2 to estimate the taxonomic richness of each sample, and we removed any sample with an error greater than one from further alpha diversity analyses (three samples: Jupiter Period 5, Lazarou Period 3, and Louie Period 2). We calculated the Shannon and Faith's Phylogenetic diversity measures using the diversity plug-in in QIIME2. To generate unweighted and weighted UniFrac distance matrices describing pairwise similarity between samples, we used the core-metrics-phylogenetic command, rarefying the data to 6,000 reads per sample.

To transform taxonomic composition data from relative abundances into count data downstream, we used quantitative PCR (qPCR) to measure bacterial 16S rRNA gene abundance in genomic DNA samples as previously described (Nadkarni et al., 2002).

Data analysis

We used mixed effects models to test for changes in fecal consistency and parasite burdens. We included trial period and clay type as fixed effects, and individual identity and species as random effects. Due to limited power, we could not include macaque species, age, or sex in these analyses, even though these factors may affect the gut microbiome (Amato et al., 2014; Amato et al., 2019). We also constructed contrasts to compare the mean fecal consistency and parasite burdens between pre-supplementation trial period 1 versus the average of other trial periods, and between supplementation trial period 2 and the average of the three post supplementation trial periods.

To test for significant differences in gut bacterial community composition in response to clay supplements, we ran a permutational analysis of variance (PERMANOVA) using the adonis function in the R package vegan (Oksanen et al., 2019), for both the unweighted UniFrac and weighted UniFrac distance matrices. We tested for the effect of both trial period and clay type after accounting for the variation explained by both monkey species and individual identity. To test for significant changes in gut bacterial diversity across species, we used linear mixed effects models with trial period and clay type included as fixed effects, and individual identity and species included as random effects. We could not include age and sex as factors in these models due to limited power. To test for differences in the relative abundances (or relative proportions) of specific microbial taxa across trial periods and between clay types, we used linear mixed

effects models on the arcsin square root transformed data at the genus and ASV level separately. The same factors described for microbial diversity were included in these models. We also constructed contrasts to compare the mean microbial relative abundance between pre-supplementation versus the average of other trial periods, and between supplementation periods and the average of three post supplementation periods. We corrected the resulting p values for multiple tests using Benjamini and Hochberg's FDR controlling procedure at significance level a = 0.05 (Benjamini & Hochberg, 1995). Except for the PERMANOVA, all tests were performed using PROC MIXED in SAS Studio.

Results

Clay minerals consumed

The monkeys differed in their behavioral responses to clay mineral supplementation, and they consumed varying amounts. Some readily ate the clay, while others spent more time investigating the clay in their hands (Figure 1). Monkeys consumed clay on an average of 3.9 (±0.9) days and consumed an average of 77% (±17%) of the clay offered to them. The monkeys showed clear preferences for different vehicles used to convey clay minerals, but in general peanut butter and banana were preferred (Table S2). As well, the monkeys preferred kaolinite over montmorillonite.

Figure 1. Examples of monkey behavioral responses to clay minerals.

Clay's effect on fecal consistency

During the clay supplementation, no monkeys displayed signs of illness. However, trial period had a significant effect on fecal consistency ($F_{4,59} = 5.5$, $p < 0.001$; Table S3). Contrasts revealed that samples collected during the three post-supplementation trial periods were softer on average than samples collected during supplementation (trial period 2). This pattern was driven by trial period 3, in particular (Figure 2; Table S4). However, all samples were considered 'normal' throughout the experiment. The effect of supplementation on fecal consistency did not vary by clay type ($F_{1,59} = 2.3$, $p = 0.13$; Table S5).

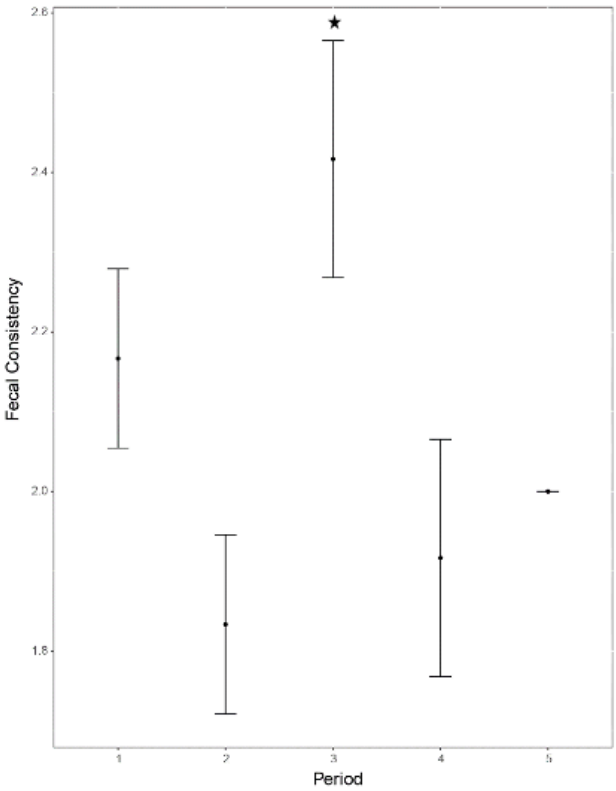

Figure 2. Average fecal consistency (± SE) of monkeys during each of the five study periods (before clay administration, during, and for three weeks after). Consistency was evaluated according to the following study-specific scale within the normal range (2-3) of the Bristol stool scale: 1=firm, 2= normal, 3=soft.

Clay's effect on parasites

Gastrointestinal parasite prevalence varied minimally in response to clay supplementation. Both *Iodamoeba butschlii* and *Entamoeba* sp. were detected during trial period 1 before clay supplementation (Table S6). The prevalence of *I. butschlii* was significantly lower during pre-supplementation trial period 1 compared to all other trial periods as well as during supplementation (trial period 2) compared to the subsequent post-supplementation trial periods ($F_{4,59} = 7.1$, $p < 0.001$), while the prevalence of *Entamoeba* sp. did not change significantly ($F_{4,59} = 1.0$, $p = 0.42$; Tables S3, S4). Clay type had no effect on these results (*I. butschlii*: F1,59 = 0.76, p = 0.39; *Entamoeba* sp: $F_{1,59} = 0.28$, $p = 0.60$; Table S4).

Clay's effect on the microbiome

After accounting for the effect of individual identity and species, trial period had a significant, albeit minor, effect on overall gut microbiome composition (unweighted UniFrac: pseudo-$F_{4,69} = 1.3$, $r^2 = 0.05$, $p < 0.01$; weighted UniFrac: pseudo-$F_{4,69} = 2.2$, $r^2 = 0.08$, $p < 0.001$; Fig. 3). Clay type had no influence on these results (interaction between trial period and clay type: unweighted UniFrac: pseudo-$F_{4,69} = 1.1$, $r^2 = 0.04$, $p = 0.24$; weighted UniFrac: pseudo-$F_{4,69} = 1.1$, $r^2 = 0.03$, $p < 0.28$). There were no differences in gut microbial diversity as a result of the trial period (Tables S3, S4), but macaques that receive kaolinite had slightly higher Shannon diversity ($F_{1,47} = 9.2$, $p = 0.004$; Table S5). The relative abundances of 27 microbial ASVs and 35

genera varied statistically in response to the trial period (Tables S7, S8). Of these, only five ASVs and eight genera demonstrated significant differences between pre-supplementation (trial period 1) and all other periods or between supplementation (trial period 2) and post-supplementation (trial periods 3, 4, and 5; Tables S9, 10). For example, the relative abundances of a *Roseburia* and an unknown Bacteroidales family p-2534-18B5 strain temporarily increased in response to supplementation ($F_{4,44} = 6.2$, $p < 0.01$; F4,44 = 6.1, $p < 0.01$; Fig. S1, S2), while the relative abundance of an *Anaeroplasma* strain increased stably ($F_{4,44} = 5.1$, $p < 0.01$, Fig. S3). At the genus level, the relative abundances of an unknown Bacteroidetes genus, and an unknown S24-7 genus, *Rummeliibacillus*, and two unknown Clostridia genera increased (Figs. S4-8), while the relative abundances of *Porphyromonas* and an unknown Paraprevotellaceae genus decreased (Figs. S9, 10). Clay type had a significant effect on the relative abundances of 36 microbial ASVs and 24 microbial genera (Table S11, S12).

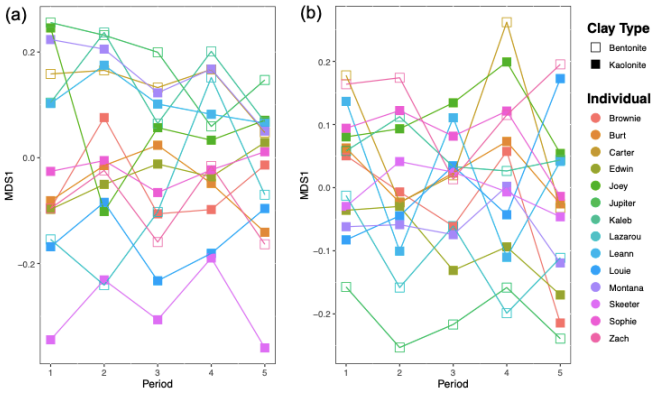

Figura 3. Non-metric multidimensional scaling (NMDS) plot illustrating changes in the gut microbiome composition of the macaque gut microbiome across each study period (before clay administration, during, and for three weeks after) using (a) unweighted UniFrac distances and (b) weighted UniFrac distances. Time period 1 is pre-supplementation. Time period 2 is during clay supplementation, and time periods 3-5 are after supplementation.

Discussion

In this pilot study, we tested whether healthy captive macaques would consume clay supplements and examined the influence of clay consumption on multiple short-term measures of gastrointestinal health. In general, we found that the monkeys consumed the clay supplements, although there were clear preferences for the delivery mode and the type of clay mineral, with some variation in the amount consumed. We observed slight changes in fecal consistency, parasite burdens, and gut bacterial community composition across trial periods, which were potentially in response to clay supplementation. However, these changes were minimal and unlikely to affect short-term health. Although the monkeys preferred kaolinite to montmorillonite, contrary to predictions, the type of clay utilized did not affect any outcome. Our findings suggest that clay supplementation is safe to test in a range of species and health contexts to better understand its physiological impacts. Furthermore, monkey behavioral responses to clay suggest it may be a useful enrichment tool.

Our study suggests that, once consumed, clay slightly altered the consistency of the stool, and most monkeys maintained a normal stool consistency throughout the study. This outcome did not depend on the type of clay consumed, suggesting that whatever the monkeys prefer is likely to result in similar outcomes. That being said, clay supplementation should be used with care. During our study, three individuals demonstrated "clay-like" feces, indicative of constipation risk. This development was likely a result of excess clay consumption. Two of the individuals had received 4-5 doses of kaolinite, and one had received four doses of montmorillonite compared to fewer doses in other individuals. Given that clay remains in the gastrointestinal tract for several days, we suggest that subsequent studies trial different doses and consider providing clay supplementation every other day instead of every day. Additionally, regardless of the dosing schedule, water should always be available, and feces should be monitored so that animals do not become constipated. Clay supplementation should also be tested in the context of disease, particularly in animals with diarrhea that might benefit from reduced water content in stool.

Clay supplementation also had minimal effects on parasite prevalence. *Entamoeba coli* prevalence remained low throughout the study, and while *Iodamoeba butschlii* parasite prevalence appeared to increase with clay supplementation, potential confounds with sampling frequency must also be considered. At Primarily Primates, individuals are generally tested for parasites quarterly, and pre-supplementation samples represented one of these quarterly timepoints. For the rest of the study, individuals were sampled for gastrointestinal parasites weekly. More frequent sampling can increase estimates of parasite prevalence in various contexts (Villanúa et al., 2006), and we believe this is what drove the increased prevalence of gastrointestinal parasites in our study. Alternatively, it is possible that clay consumption increased retention time and reduced parasite turnover during the study. This dynamic could also lead to increased parasite prevalence. Although follow-up research is necessary to further test these hypotheses, we believe that the actual effect of clay on parasite prevalence was neutral.

Finally, clay supplementation did not appear to have any biologically significant effects on the gut bacterial composition, regardless of clay type. While some statistically significant patterns emerged, they were primarily associated with a small number of low-abundance microbial taxa. Therefore, the biological impact of these changes is likely to be minimal. Little prior data exist on the impact of clay supplementation on the gut bacterial community. Clay has been reported to adsorb some pathogenic bacteria (e.g., *Pseudomonas aeruginosa*, *Escherichia coli*) (Said et al., 1980; Vondruskova et al., 2010), but these taxa were not present in high relative abundances in our healthy macaques. Clay supplementation may have little impact on the normal gut bacterial community, particularly when these pathogens are not present. Alternatively, our inability to control for macaque species, age, sex, reproductive

status, and previous research use may have contributed to additional noise in the microbiome data, since many of these factors are likely to affect the microbiome (Amato et al., 2019). Furthermore, microbial functional genes may exhibit more fine-scale shifts in response to clay supplementation than microbial taxonomic composition. More information examining the specific strains and functional gene profiles of the microbiome are necessary to improve insight into the potential influence of clay supplementation on the microbiome.

Overall, our results suggest that clay supplementation should be more seriously considered as a low-risk alternative to antimicrobial, steroidal, and antidiarrheal drugs. Although we did not test its efficacy in diseased individuals, it does not appear to have short-term negative health impacts on healthy macaques. Future studies should conduct longer trials to evaluate whether clay supplementation poses any long-term threats to GI health and evaluate whether clay minerals can serve as a novel treatment for primates suffering from chronic diarrhea. Using this pilot study as a foundation, we suggest that controlled, longitudinal studies should be developed to examine the relative efficacy of clay supplementation, antimicrobial, steroidal, and antidiarrheal drugs. There should also be an additional examination of appropriate doses as well as the specific mechanisms through which clay affects host physiology, gastrointestinal parasites, and the gut bacterial community.

Importantly, one of the first concerns testing the health effects of clay supplements is getting primates to consume them. Our experiences suggest that selecting the correct vehicle for clay supplementation is critical. In captive contexts, if caregivers are aware of favorite foods, that is the ideal way to offer them. Following their food preferences more generally, the monkeys at Primarily Primates preferred clay in either peanut butter or bananas. Other populations will have different preferences. For example, in a short pilot study on clay supplementation among chimpanzees the Edinburgh Zoo (Royal Zoological Society of Scotland), most individuals readily ate clay when mixed with oats and water (Pebsworth, unpublished data). In the wild, monkeys consume soil without a vehicle, which contains not only clay but also silt and a small percentage of sand. Ideally, supplementation would resemble these soils, not 100% clay. In this sense, the type of clay is likely to influence both the vehicle and the monkeys' propensity to consume it. Montmorillonite has a slight smell, so it may be harder to conceal than kaolinite. Additionally, when moistened, montmorillonite becomes unctuous and, depending on what it is mixed with, may have an off-putting mouthfeel. In our study, several monkeys refused to eat it (Figure 1). This behavior differed by the vehicle used. Because montmorillonite is a swelling clay, it readily adsorbed the liquid in applesauce, making it very thick, and few monkeys consumed it when delivered this way. In contrast, only two monkeys refused the montmorillonite when it was mixed with peanut butter.

Finally, although we did not collect quantitative behavioral data in this study, we did observe that several monkeys spent time exploring how clay felt in their hands. This behavior was most often observed in monkeys that were offered montmorillonite. For these individuals, clay supplementation may serve as a low-cost behavioral stimulation or form of enrichment, thereby conferring benefits beyond those potentially associated with clay consumption.

Conclusions

More research is needed, but our pilot study suggests that clay minerals do not have negative impacts on the short-term health of healthy captive macaques. Because of these neutral effects, there are few clear risks to further exploring the physiological effects of clay consumption in primates. Our findings suggest that kaolinite should be used as a preferred clay since it is easier to administer than montmorillonite and appears to have the same effect. However, we suggest that future research test the effects of kaolinite on both healthy individuals and those with GI disease in a more extended longitudinal study with a population in which more co-variates can be controlled. It should also generate data for a larger range of primate species and dosing schedules. Finally, the incorporation of additional health measures will provide further insight into the potential benefits and risks of clay consumption by primates.

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