Impact of stress on the gut microbiome of free-ranging western lowland gorillas

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Abstract

Exposure to stressors can negatively impact the mammalian gastrointestinal microbiome (GIM). Here, we used 454 pyrosequencing of 16S rRNA bacterial gene amplicons to evaluate the impact of physiological stress, as evidenced by faecal glucocorticoid metabolites (FGCM; ng/g), on the GIM composition of free-ranging western lowland gorillas (Gorilla gorilla gorilla). Although we found no relationship between GIM alpha diversity (H) and FGCM levels, we observed a significant relationship between the relative abundances of particular bacterial taxa and FGCM levels. Specifically, members of the family Anaerolineaceae (p=0.4, FDR q=0.01), genus Clostridium cluster XIVb (p=0.35, FDR q=0.02) and genus Oscillibacter (p=0.35, FDR q=0.02) were positively correlated with FGCM levels. Thus, while exposure to stressors appears to be associated with minor changes in the gorilla GIM, the consequences of these changes are unknown. Our results may have implications for conservation biology as well as for our overall understanding of factors influencing the non-human primate GIM.

The gastrointestinal microbiome (GIM) is interconnected with overall host health [1, 2]. Disruptions in the GIM have been associated with a large array of human diseases, such as inflammatory bowel disease [3], allergies, asthma [4, 5], neurodevelopmental illnesses [6] and obesity [7]. Stress, which is defined as an acute threat to homeostasis, evokes adaptive or allostatic responses and can have both short- and long-term influences on the gastrointestinal (GI) tract. Activation of these adaptive or allostatic systems can become maladaptive because of frequent, chronic or excessive stress, leading to predisposition to disease [8]. The major effects of stress on gut physiology include: (1) alterations in GI motility; (2) increase in visceral perception; (3) changes in GI secretion; (4) increase in intestinal permeability; (5) impaired regenerative capacity of GI mucosa and mucosal blood flow; and (6) negative effects on the GIM [9]. Chronic and acute stress models are widely employed in GI research, because stress has been identified as a risk factor for or modulator of the expression of several GI disorders [9–11].

Many factors, including exposure to stressors, can cause transient alterations in GIM composition [12–16]. Moreover, stressor-induced changes to the GIM may enhance the ability of enteric pathogens to colonize the intestine [17]. Indeed, stress can influence the outcome of bacterial infections, because enteric bacteria can respond to the release of stress-related neurochemical mediators by the host [18]. Reductions in bacterial counts have been observed in...
human GIMs due to stress [19], as well as in infant rhesus monkeys, where a significant decrease in lactobacilli was recorded in response to stressors [20]. To date, the relationship between stress and the GIM has mainly been studied in humans, or in the laboratory, or in captive animals, and it has mostly been examined through qualitative evaluation of stressors [17, 21]. As part of a long-term monitoring programme of a population of western lowland gorillas [Gorilla gorilla gorilla (WLG)] in the Dzanga Sangha Protected Areas (DSPA), Central African Republic (CAR), we previously determined their faecal glucocorticoid metabolite (FGCM) levels, a proxy for physiological stress that shows differences related to the level of habitation to the presence of humans [22]. We also characterized the faecal bacterial communities in this DSPA gorilla population [23, 24]. Here, we evaluate the relationship between FGCM levels and GIM composition on a set of faecal samples from four WLG groups in DSPA characterized by different levels of habitation to humans and thus exposed to different levels of physiological stress. The research adhered to the legal requirements of the CAR and DSPA research protocols. Importation of the samples to the EU was approved by the State Veterinary Authority of the Czech Republic.

Faecal samples were collected from two habituated groups at two different DSPA research sites: H1 (n=9) and H2 (n=12) at Bai Hokou (2° 50’ N, 16° 28’ E) and Mongambe (2° 55’ N, 16° 23’ E), respectively. Faecal samples from two unhabituated groups were also collected: U1 (n=10) near Bai Hokou (an unhabituated group undergoing habituation) and U2 (n=11, an entirely wild group) at a site between two research sites (Fig S1, available in the online version of this article; for more details about the groups see Shutt et al. [22]). Samples were collected from June to September 2011. Samples from habituated WLGs were obtained during gorilla follow, while unhabituated, unidentified WLGs were sampled from their nests to avoid duplication. Only fresh faeces were collected, that is, samples were taken within 3 h from the time we assumed the WLGs had left the nests, as faeces were collected, that is, samples were taken within 3 h and U2 (n=12) at Bai Hokou (2° 50’ N, 16° 28’ E) and Mongambe (2° 55’ N, 16° 23’ E), respectively. Faecal samples from two unhabituated groups were also collected: U1 (n=10) near Bai Hokou (an unhabituated group undergoing habituation) and U2 (n=11, an entirely wild group) at a site between two research sites (Fig S1, available in the online version of this article; for more details about the groups see Shutt et al. [22]). Samples were collected from June to September 2011. Samples from habituated WLGs were obtained during gorilla follow, while unhabituated, unidentified WLGs were sampled from their nests to avoid duplication. Only fresh faeces were collected, that is, samples were taken within 3 h from the time we assumed the WLGs had left the nests, as per expert tracker assessment. Each individual was sampled noninvasively and was only sampled once. The samples were taken from within the core of the faecal bolus and stored in RNAlater (Thermo Fisher Scientific, Waltham, MA, USA) for microbiome analyses, and 90% ethanol for FGCM analysis. Validated methods were followed to avoid variation in our FGCM measurements resulting from sampling, extraction or storage conditions [25]. The samples were shipped to the German Primate Centre Endocrinology Laboratory (Göttingen, Germany) for hormone analysis. The samples for microbiome analyses were kept at room temperature for a maximum of 1 month before being transported to the Department of Pathology and Parasitology, University of Veterinary and Pharmaceutical Sciences Brno, Czech Republic, where they were stored at −20 °C until they were shipped to the Institute for Genomic Biology, University of Illinois at Urbana-Champaign, USA, where the genomic DNA extractions were performed. The resulting genomic DNA was shipped to the J. Craig Venter Institute, Rockville, MD, USA, where DNA sequencing was performed.

DNA was extracted from the faecal samples using the Power Soil DNA isolation kit (MoBio Laboratories, Inc., Carlsbad, CA, USA) according to the manufacturer's protocol. We purified obtained DNA using the QIAquick gel extraction kit (QIAGEN, Germany). The V1–V3 region of the 16S rRNA gene was amplified (35 cycles: at 95 °C for 30 s, at 55 °C for 30 s and at 72 °C for 30 s) using the primers 27f (5'-AGAGTTTGATYMTGGCTCAG-3'), corresponding to nucleotides 27–47 of the Escherichia coli 16S rRNA gene) and 534r (5'-ATTACCGCGGTGTGCTGCGA-3', corresponding to nucleotides 534–515 of the E. coli 16S rRNA gene). The amplicons were multiplexed and pyrosequenced using 454 FLX-Titanium technology as described in [23]. Briefly, the sequence reads were processed using the online tool mothur and its standard 454 SOP [26]. Unique sequences were aligned against the SILVA reference alignment database and chimeras were detected using uchime [27] and removed. The sequences were then classified taxonomically using a Bayesian classifier approach implemented by mothur and reference files from the Ribosomal Database Project (RDP) [28] with a minimum cut-off of 80%. Then sequence reads with hits corresponding to unknown, mitochondria, chloroplasts, eukaryotes and archaea were eliminated. The remaining reads were clustered de novo using ModalClust. Reads sharing ≥97% 16S rRNA sequence complete-linkage similarity with the most abundant sequence were binned into an operational taxonomic unit (OTU). Taxonomic profiles were determined using the RDP classifier [28] and the phyloype function within mothur. OTUs detected fewer than five times across the entire data set, and/or in fewer than three (in one and two), individuals were removed to avoid including probable sequence artifacts in the analyses.

FGCM measurements were performed using a 11β-hydroxyetiocholanolone (3α,11β-dihydroxy-CM) enzyme immunoassay as previously validated in Shutt et al. [25]. The inter-assay coefficients of variations for these measurements were 9.2% (high-value quality control) and 15.1% (low-value quality control). Any samples with known complications (e.g. seeds discovered in the faecal matrix or alcohol evaporation) were removed. We express all hormone data as hormone content per faecal wet mass (ng/g).

All statistical analyses were performed in R v3.1.2 [29]. We used the stats package to perform linear regression models [30]; the psych package [31] for Spearman rank correlations used with false discovery rate analyses; the ggplot2 package [33] to plot the PCoA with colour key.
The median of the FGCM levels (FGCM/g of faeces) was 82.47 (min, 26.74; max, 243.61); for more details see Shutt et al. [22]. We did not find significant differences in FGCM levels among gorilla groups ($\chi^2=6.64, P=0.08$, Kruskal–Wallis rank sum test). We obtained a median value of 6495 16S rRNA pyrosequencing reads per sample (min, 2144; max, 116 361) after sequence quality control; for more details see [23, 24]. The GIM profiles of the major and minor phyla detected in each gorilla group are shown in Fig. S2. We observed significant differences in GIM profiles among the studied gorilla groups (pseudo $F=2.62, P<0.01$, PERMANOVA; Fig. S3). A PCoA plot showed that the GIM composition in the four WLG groups did not vary according to FGCM levels, with the exception of group U2, where individual samples clustered together (Fig. 1a). U2 was the group with the lowest FGCM levels (Fig. 1a, b). Although we observed variation in the GIM composition profiles among the groups, based on the first principal coordinate and FGCM levels, with individuals in U2 showing the lowest stress hormone metabolite values, this relationship was not significant (linear regression model: adjusted $R^2=0.01, t=-1.22, P=0.23$; Spearman rank correlation: $\rho=-0.24, P=0.12$; Fig. 1c). Nevertheless, we did observe significant relationships between the relative abundances of particular bacterial genera and FGCM levels across the studied gorilla groups. Namely, members of the family Anaerolineaceae (linear regression model: adjusted $R^2=0.12, t=2.55, P=0.02$; Spearman rank correlation: $\rho=0.4, \text{FDR } q=0.01$), the genus Clostridium XIVb (linear regression model: adjusted $R^2=0.18, t=3.19, P<0.01$; Spearman rank correlation: $\rho=0.35, \text{FDR } q=0.02$) and the genus Oscillibacter (linear regression model: adjusted $R^2=0.19, t=3.21, P<0.01$; Spearman rank correlation: $\rho=0.35, \text{FDR } q=0.02$) were positively correlated with FGCM levels (Fig. 2). We did not find any differences in the relative abundances of these bacterial taxa across the entirety of the studied WLG groups, with the exception of Oscillibacter between U1 and U2 ($P=0.05$, Kruskal–Wallis tests adjusted for multiple comparisons, Fig. S4). Likewise, no significant relationship was detected between the relative abundance of other bacterial taxa and FGCM levels (data not shown). We also did not observe any correlations between GIM alpha diversity (Shannon diversity indices) and FGCM levels (linear regression model: adjusted $R^2<0.01, t=-0.03, P=0.98$; Spearman rank correlation: $\rho=0.03, P=0.86$; Fig. S5).

These results suggest that stress has minimal impact on the overall composition of the GIM in the studied WLGs, with the exception of individuals from group U2, whose GIM clustered together and showed the lowest FGCM levels. We did not observe significant differences in FGCM levels among the studied groups, which is in contrast to Shutt et al.’s findings [22], however, this study used a large sample set. The specific changes in the abundance of particular bacterial taxa along with FGCM levels may be noteworthy. The taxa correlated with FGCM levels have been consistently found in the GIM of great apes, and could be considered to be normal commensals in non-human primates [23, 34, 35]. Higher abundance of Oscillibacter has also been observed in humans on a resistant-starch and reduced-carbohydrate weight-loss diet, and is depleted in patients suffering from Crohn’s disease [36, 37]. Members of the genus Clostridium from the cluster XIV with sub-clusters

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**Fig. 1.** Relationship of GIM composition and FGCM levels of individual WLGs from four groups. U1 and U2, unhabituated groups; H1 and H2, habituated groups. (a) PCoA plot of GIM (described with first and second principal coordinates) with colour key representing FGCM levels. (b) Box plots of natural log-transformed FGCM levels in the studied WLG groups; mean values are represented by lines in the boxes. (c) Linear regression model of GIM (first principal coordinate) and FGCM levels (natural log-transformed).
XIVa and XIVb [38] include several cellulolytic *Clostridium* species that decompose and ferment various polysaccharides and disaccharides derived from plant cell walls [39]. Increases in the relative abundances of *Clostridium* spp. have also been observed in adult mice due to social stressors [21]. Unlike the clostridia detected in our study (cluster XIV), these bacteria include pathogenic *Clostridium* species known to induce inflammation [40]. Thus, it is possible that changes in the abundance of these taxa upon exposure to stressors are confounded with different dietary behaviours within these WLG groups due to small-scale geographical separation and socio-ecological reasons, such as group size difference [23, 41]. Moreover, previous studies showed that the presence of humans and the habitation process have an effect on gorilla feeding behaviour. For the group undergoing habitation, the daily path lengths were longer during the early stages of habitation, when the gorillas were avoiding observers [42], which may have an indirect impact on feeding behaviour. In habituated groups, when the number of visiting people increased, the silverbacks (at least) spent more time monitoring humans and less time feeding [41]. Future studies should thus clarify if the stress affects feeding behaviour, thereby changing the microbiome, or if there is a direct effect on the microbiome composition and function. Furthermore, as these bacterial taxa are poorly characterized in the non-human primate gut, it is hard to speculate concerning the combined effect of FGCM (stressors) and ecological factors (diet) on the abundance of these taxa in the gut of habituated and unhabituated WLGs.

Our results show that stressors, as evidenced by elevated FGCM levels, may have minor, specific effects on the overall GIM composition of free-ranging WLGs. Specifically, we observed that higher FGCM levels are associated with higher levels of members of the family *Anaerolineaceae*, genus *Clostridium* cluster XIVb and genus *Oscillobacter*. However, it is unclear whether these minor changes have a significant impact on the gut microbial communities at large (e.g. dysbiosis) or on overall host health. Although a completely unhabituated group (U2) showed the lowest incidence of physiological stress, and the GIM compositions among individuals from this group were more similar to one another than those observed in the other studied groups, it is unclear whether the individuals with higher exposure to stressors (i.e. having the highest FGCM levels), namely those from the H1, H2 (both habituated) and U1 (undergoing habitation) groups, exhibit significant changes in their GIM composition. Our data do not support the notion that stress linked with the habitation of wild apes to humans has major effects on their GIM. However, any potential changes may be the result of several ecological and dietary factors acting together over long periods of time. It is important to control for diet-induced GIM changes within each studied gorilla group. To further assess the effect of stressors on the GIM of wild non-human primates, long-term multivariate studies that monitor fluctuation in FGCM levels together with changes in GIM profiles over the entire habitation process, in one gorilla group, are warranted.

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**Conflicts of interest**

The authors declare that there are no conflicts of interest.

**References**


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