Supplementary material S1 for "*Context-dependent effects of glucocorticoids on the lizard gut microbiome"*, MacLeod KJ, Kohl KD, Trevelline BK, Langkilde T. (Molecular Ecology 2021)

Full molecular protocols

1. DNA extraction and amplification

We isolated DNA from fecal samples using the Qiagen PowerFecal DNA Kit (Qiagen, Hilden, Germany; product number: 12830) with an overnight incubation in lysis buffer at 65°C to increase extraction yields (Trevelline 2018). We also conducted four 'blank' extractions to correct for contaminants found in DNA extraction kits (Salter et al., 2014). We used polymerase chain reaction (PCR) to amplify a portion of the bacterial 16S rRNA gene for Illumina sequencing using the primers 515F and 806R (modified from the primer set employed by the Earth Microbiome Project (EMP; GTGCCAGCMGCCGCGGTAA and GGACTACNVGGGTWTCTAAT) targeting the V4 region of microbial small subunit ribosomal RNA gene (Caporaso et al., 2011). All primers contained 5′ common sequence tags (known as common sequence 1 and 2, CS1 (ACACTGACGACATGGTTCTACA) and CS2 (TACGGTAGCAGAGACTTGGTCT) as described previously (Moonsamy et al., 2013), with forward primers containing the CS1 linker and reverse primers containing the CS2 linker. Amplicons were generated using a two-stage targeted amplicon sequencing (TAS) protocol (Bybee et al., 2011; Green et al., 2015). First-stage PCR amplifications of 16S rRNA gene fragments were performed in 10 μL reactions in 96-well plates, using the MyTaq HS 2X mastermix (Bioline, London, UK; product number: 25045) and the following thermal cycling conditions: 95°C for 5 minutes, followed by 28 cycles of 95°C for 30 seconds, 55°C for 45 seconds and 72°C for 30 seconds.

Subsequently, a second PCR amplification was performed in 20 µL reactions in 96-well plates using the MyTaq HS 2X mastermix. Each well received a separate primer pair with a unique 10-base barcode, obtained from the Access Array Barcode Library for Illumina (Fluidigm, South San Francisco, CA; product number: 100-4876). These AccessArray primers contain the CS1 and CS2 linkers at the 3′ ends of the oligonucleotides and can be used without any specific Fluidigm equipment. Thermalcycling conditions were as follows: 95°C for 5 minutes, followed by 8 cycles of 95°C for 30 seconds, 60°C for 30 seconds, and 72°C for 30 seconds. A final, 7-minute elongation step was performed at 72°C.

2. Amplicon library preparation and Illumina sequencing

Amplified products were pooled in equal volume using an EpMotion5075 liquid handling robot (Eppendorf, Hamburg, Germany). The pooled library was purified using an AMPure XP cleanup protocol (0.6X, vol/vol; Agencourt, Beckmann-Coulter) to remove fragments smaller than 300 bp. The pooled libraries, with 20% phiX, were loaded onto an Illumina MiniSeq mid-output flow cell (2x153 base paired-end reads). Based on the distribution of reads per barcode, the amplicons were re-pooled to generate a more balanced distribution of reads. The re-pooled library was purified using AMPure XP cleanup, as described above. The re-pooled bacterial libraries were loaded onto a second MiniSeq flow cell and sequenced (2x153 base paired-end reads). In all cases, Fluidigm sequencing primers, targeting the CS1 and CS2 linker regions, were used to initiate sequencing. Data from the two runs were concatenated before analysis. All library preparation, pooling, and sequencing was performed at the DNA Services facility at the University of Illinois - Chicago. Sequence reads have been deposited in the NCBI SRA database under PRJNA491710.

3. Illumina sequencing and bioinformatics

We sequenced bacterial 16S rRNA amplicons from a total of 142 fecal samples from 4 DNA extraction kit controls (Table 1). Sequence reads were filtered and processed using the DADA2 pipeline (Callahan et al., 2016) in QIIME2 version 2018.8 (Bolyen et al., 2018). We identified bacterial 16S rRNA sequence variants (hereafter Amplicon Sequence Variants or ASVs) using the Greengenes reference database (DeSantis et al., 2006). We removed sequences that were identified as archaea, chloroplasts, and mitochondria, and contaminant ASVs (those detected in DNA extraction kit controls). We rarefied ASV tables to 1,980 sequences before comparisons of alpha (ASV richness, evenness, Faith's phylogenetic diversity, and Shannon Index) and beta diversity (Lozupone & Knight, 2005) in QIIME2 (Bolyen et al., 2018).

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