Supplementary Materials for

NEC-like intestinal injury is ameliorated by *Lactobacillus rhamnosus* GG in parallel with SIGIRR and A20 induction in neonatal mice

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METHODS:

Mice: Time-pregnant C57BL6 mice were obtained from Charles River (Burlington, MA) and allowed to deliver naturally. Control pups remained with their mothers while experimental pups were separated from their mothers and maintained in a temperature- and humidity-controlled incubator. All animal experiments (**Supplemental Fig S1, online**) were approved by the local Institutional Animal Care and Usage Committee (Protocol 1601).

Lactobacillus rhamnosus: Freeze-dried LGG was obtained from American Type Culture Collection (ATCC 53103, Manassas, VA) and grown per manufacturer recommendations. LGG in culture media was then harvested by centrifugation (3400x *g* for 10 min) and resuspended in PBS/0.1% gelatin. LGG quantification was performed by adjusting the optical density at 600 nm to the desired concentration prior to gavage administration. Mice receiving LGG were given 0.1 mL of either low-dose LGG (10^7 CFU/mL) or high-dose LGG (10^8 CFU/mL). The number and viability of lactobacilli were confirmed by culturing on MRS plates prior to each administration.

Formula-feeding: P5 pups were randomly assigned into five groups: breastmilk-fed controls (BM), breastmilk-fed with low-dose LGG (BMP), formula-fed (F), formula-fed with low dose LGG (FP), and formula-fed with high dose LGG (FPP). Formula-fed pups were gavage-fed 0.1 mL of Esbilac canine milk replacer (PetAg, Inc., Hampshire, IL) five times daily starting at day of life (DOL) 5 to DOL 7. Pups that received LGG in the above groups were gavage-fed 0.1 mL of either low-dose LGG (10^7 CFU/mL), high-dose LGG (10^8 CFU/mL), or vehicle once daily. All pups were euthanized at DOL 8.

NEC induction: P5 pups were randomly assigned into three groups: breastmilk-fed controls (BM), NEC, and NEC with low-dose LGG (NEC-P). From DOL 5 to 7 pups were pre-treated with either LGG or vehicle as per their assigned groups. Starting at DOL 8 to DOL 10, pups in

the NEC groups underwent a modified NEC protocol as previously described (3). Mice were euthanized on DOL 11. Another set of experiments using high-dose LGG (BM, NEC, NEC-PP) instead of low-dose LGG was also conducted.

Sample collection and tissue processing: Distal colonic tissue with stool was removed and divided into (1) Carnoy's fixative to study the mucus barrier, (2) formalin for staining, and (3) snap-frozen in liquid nitrogen for 16sRNA sequencing. Distal ileum was also collected and divided into (1) formalin for staining, (2) RNA later for QT-PCR, and (3) snap frozen for Western blot studies.

qPCR. Total RNA was extracted using the RNeasy mini kit (Qiagen, Valencia, CA), and cDNA was synthesized from 1 μg of RNA using the iScript cDNA synthesis kit (Bio-Rad, Hercules, CA). The transcripts were amplified, and gene expression data were collected on a Bio-Rad 1Q5 with SYBR Green Mastermix. Pre-validated primers for 18S ribosomal RNA (18S rRNA), intercellular adhesion molecule 1 (ICAM1), mouse homolog to IL-8 (KC), and interleukin 1 beta (IL-1β) were purchased from Sigma. 18S was used as the housekeeping gene. Relative gene expression of ICAM1, KC, and IL-1β was calculated with the Pfaffl method (15).

Western blot. Clarified lysates from harvested tissue were used for protein analysis. Samples were lysed in RIPA buffer containing protease and phosphatase inhibitors (Sigma) and homogenized by the bullet blender (Midwest Scientific, St. Louis, MO). Immunoblotting was done following standard protocols. Primary antibodies for phosphorylation were as follows: rabbit anti-(p)IKKβ(Ser177), mouse anti-IKKβ, rabbit anti-(p)p65(Ser536) (Cell Signaling Technology, Danvers, MA), rabbit anti-p65 (Santa Cruz Biotechnology, Santa Cruz, CA), and mouse anti-β-Actin (Sigma). Other antibodies used were as follows: mouse anti-ICAM1, rabbit anti-IL-1β (Santa Cruz Biotechnology), rabbit anti-SIGIRR (Thermo Fisher Scientific, Rockford,

IL) and rabbit anti-Cleaved Caspase 3 (Asp175) (Cell Signaling). Blots were developed using enhanced chemiluminescence and were stripped using Restore Plus stripping buffer (Thermo Fisher, Rockford, IL). β-Actin or the corresponding non-phosphorylated antibody was used for normalization, and densitometry was performed using ImageJ Software (NIH, Bethesda, MD).

16S rRNA sequencing: Total genomic bacterial DNA was extracted using the QIAmp DNA stool kit (Qiagen) following their instructions. The integrity, concentration, and quality of the DNA were assessed by agarose gel electrophoresis, and determined by absorption at A260, and A260 to A280 ratio, respectively using a Nanodrop-2000 spectrophotometer (Thermo Fisher Scientific). Using bacterial gDNA, the V3-V4 region of the 16S ribosomal RNA (rRNA) encoding gene was amplified with barcoded universal bacterial primers followed by sequencing on Illumina MiSeq platform (Illumina, San Diego, CA). The resulting raw sequence files (.fastq.gz) were submitted to the NCBI Sequence Read Archive (SRA) database (https://www.ncbi.nlm.nih.gov/sra/SRP160909). The raw sequences were analyzed using open-source bioinformatics pipeline called Quantitative Insights Into Microbial Ecology (QIIME). Reads were trimmed and demultiplexed using exact matches to the supplied DNA barcodes. Any reads with homopolymer runs, more than 6 ambiguous bases, nonmatching barcodes, barcode errors, or quality scores less than 25 were removed. Samples with less than 3500 a sequence were also removed. Resulting sequences were searched against the Greengenes 13_5 reference sequence set and clustered at 97% by Uclust. The centroid of each Operational Taxonomic Unit (OTU) was considered as the OTU representative sequence followed by aligning the sequences with PyNast and construction of Trees with FastTree for phylogenetic calculations.

Histologic grading of intestinal injury. Two separate investigators blinded to the experimental conditions (AC and WY) graded histologic intestinal tissue samples using a 4-point scale as

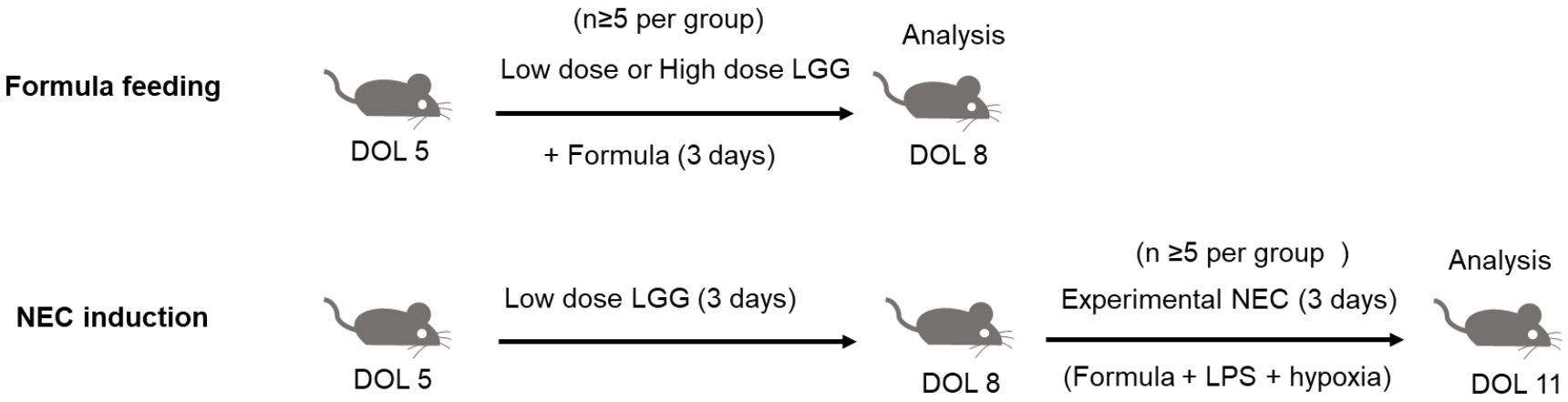
previously described (13). Slides were scanned into a computer at 40X using a Leica Biosystems Slide Scanner and the analysis was completed using the system's imaging software (Aperio ImageScope) (Leica Biosystems, Buffalo Grove, IL).

TUNEL assay for apoptosis. Terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) assay was performed on the terminal ileum slides per manufacturer instructions (Promega, Madison, WI). Images were captured at 20X using a Zeiss LSM 510 confocal microscope with an attached camera. Quantitation of the TUNEL assay was done by measuring the total number of DAPI stained cells (blue) and total number of the TUNEL positive cells (green) per high power field (HPF) using the ImageJ software (NIH), with at least 4 HPF per sample. The apoptotic index was calculated as the proportion of DAPI+ (blue) cells that stained for TUNEL+ (green).

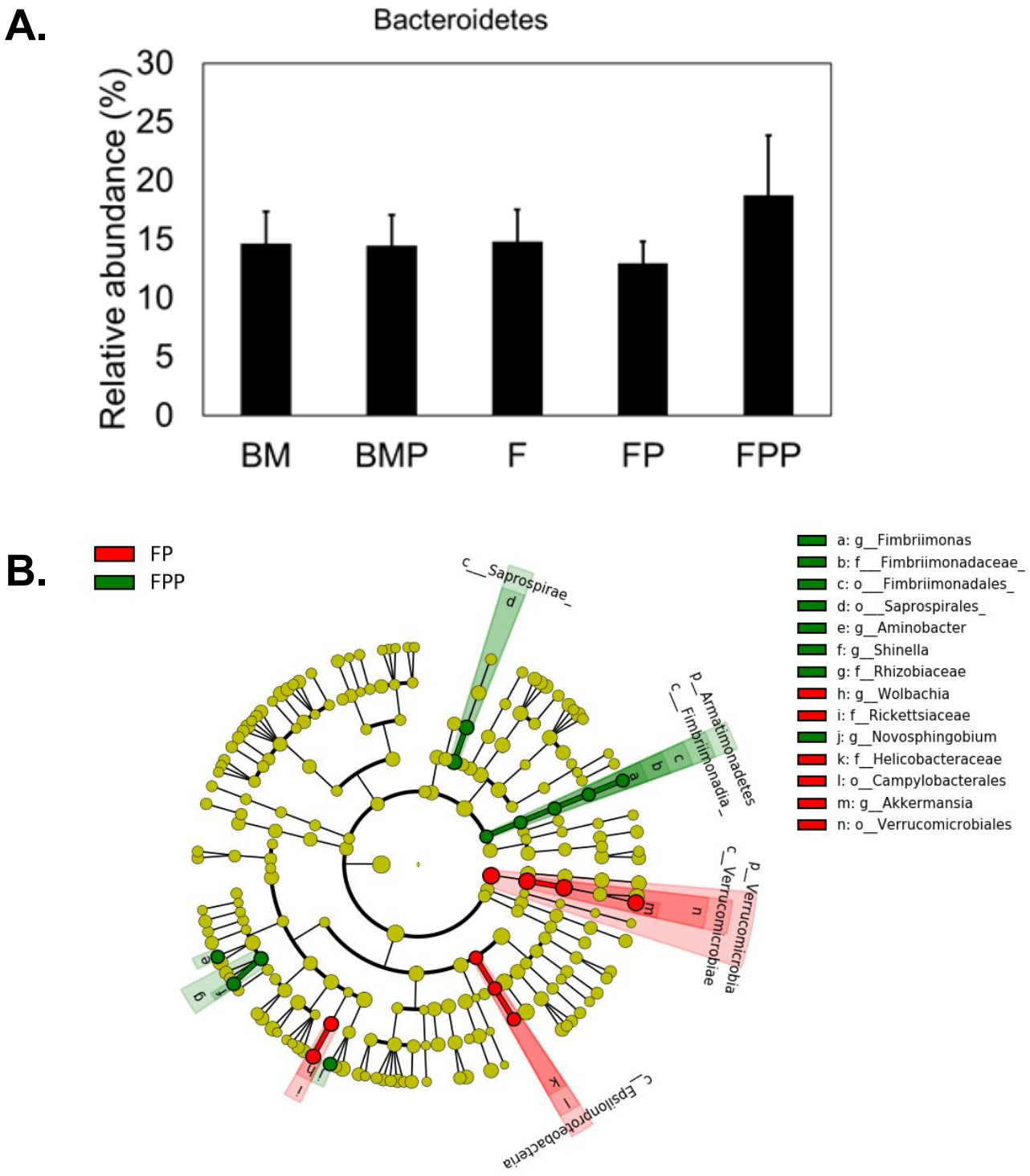
EUB338 staining. Fluorescence in situ hybridization (FISH) was performed according to the method described previously with some modification (16). Paraffin sections were dewaxed and rehydrated in an ethanol gradient to water. The tissue sections were incubated with 5 μg/ml Texas Red-conjugated EUB338 (5'- GCTGCCTCCCGTAGGAGT-3', Invitrogen) in hybridization buffer (0.1M Tris-HCl, 0.9MNaCl, 0.1% SDS and 10% formamide, pH 7.2) at 40˚C overnight. The sections were rinsed in washing buffer (20 mM Tris-HCl, 0.9MNaCl, pH 7.4) at 40˚C for 15 min and stained with 1 μg/ml DAPI. After staining, the sections were mounted with Prolong Gold mounting medium (Invitrogen). All images were obtained and analyzed with a Nikon i80 microscope.

Data Analysis. A minimum of ≥5 mouse pups were used for each experimental group. Fold changes in protein levels were determined relative to expression in control mice, and were compared between groups using ANOVA with post-hoc Tukey test used for multiple

comparisons. For changes in phosphorylation, phosphorylated to total protein ratio was calculated for each condition and compared between groups using ANOVA. For mRNA studies biological replicates were used, fold-change was calculated relative to expression in control mice, and compared between experimental groups using ANOVA. Statistical analysis was done using GraphPad Prism version 7 (GraphPad Software, San Diego, CA). Statistical significance was set at $P < 0.05$.



Supplemental Fig. S1. Flow diagram of the mouse experiments conducted in the study.



Supplemental Fig. S2. (A) Bar graphs showing relative abundance of Bacteroidetes phyla in the colonic feces of the five groups of mice (BM, BMP, F, FP, FPP) with higher levels recorded in the FPP group. **(B)** The cladogram showing significant differences in FP vs. FPP groups. Red and green indicate different groups, with the species classification at the level of class, order, family, and genus projecting from the inside.