SUPPLEMENTARY INFORMATION

Directed remodeling of the mouse gut microbiome inhibits the development of atherosclerosis

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Animals and diets.

LDL receptor-null (\textit{LDLr}^{-/-}) mice on a C57BL/6J background were initially purchased from Jackson Laboratories (Bar Harbor, ME) and were bred in house. Mice were housed in cages with paper bedding, 4 animals per cage, with 12 hour light/dark cycle. Unless otherwise noted, all experiments were performed with 8-12 weeks old LDLr^{-/-} female mice. For studies involving analysis of the gut microbiome, the mice were rotated between cages and co-housed with the mice from different cages for at least one week before the beginning of each study to reduce the variation in the composition of gut microbiome caused by the housing environment. The mice were weaned at 4 weeks of age and were fed \textit{ad libitum} a standard chow diet (Harlan Teklad 7019) until they were 10-weeks old, when they were switched to a high-fat diet (WD) containing 15.8\% (wt/wt) fat, 1.25\% (wt/wt) cholesterol, and no cholate (Harlan Teklad 94059). At the time that the WD was started, the cyclic peptide was added to the drinking water at a dose of 180 \(\mu\text{M}\) and the mice were continued on WD for 10 weeks. The lyophilized peptide was dissolved in PBS (pH 7.4) containing 1\% sucrose, resulting in a clear solution. The drinking water solution was replaced with fresh solution daily for \textit{c[wLwReQeR]} and \textit{c[w^MeLwR^MeQeR]} or every other day for \textit{c[wLwKhShK]} and \textit{c[fWwYqHhQ]}. A control group of eight mice (female, \(n=8\)) was fed WD and provided vehicle (PBS with 1\% sucrose) drinking water. The mice each consumed approximately 4.0 mL of water per day, and there was no significant difference in water or food consumption between groups. The 10-wk efficacy studies were carried out twice for both \textit{c[wLwReQeR]} and \textit{c[wLwKhShK]}. The first studies used standard, non-sterilized diet and handling conditions. To lessen the possibility that bacteria from the environment would affect the gut microbiota composition during the study, in the second set of 10-wk efficacy studies we sought to
prevent the introduction of exogenous bacteria to the mice. These studies involved sterilized
diet (irradiated LabDiet 5053, UV-irradiated by the manufacturer), sterilized
cages/bedding/water bottles (autoclaved as a unit prior to cage changes), and cages were
only opened inside a HEPA filtered animal transfer station for cage changing or to take
mouse samples. No differences were observed in the effects of the peptides on plasma total
cholesterol levels or atherosclerotic lesions between the standard handling and “sterile”
studies. For various follow up mechanistic studies, additional, shorter two- or four-wk studies
were carried out for c[wLwReQeR] (including one study conducted by different personnel at a
different facility with mice from a different colony, at Bristol-Myers-Squibb in Hopewell, NJ); in
each of these studies we observed the same degree of reductions in plasma total cholesterol.

Power analysis: For the 2-week plasma cholesterol levels and 10-week plasma cholesterol
and atherosclerotic lesion levels, differences of ≥30% between groups is typical, and the
standard deviation within groups is typically less than 25% from the mean. Based on these
values, a Power analysis indicates that our group size of n=8 animals/group is sufficient to
detect statistically significant changes (alpha = 0.05) with >90% probability. All procedures
involving live animals were approved by the Scripps Research Institute Institutional Animal
Care and Use Committee.

**Cyclic peptide synthesis and preparation.**

Peptides were synthesized by using standard Fmoc chemistry with an Advanced Chemtech
Apex 396 peptide synthesizer. A typical synthesis was performed on 0.09-mmol scale using
0.6 mmol/g Rink amide MBHA resin loaded with Fmoc-Glu-OAll via the side chain to yield a
Gln residue after cleavage from the resin, or loaded with Fmoc-Lys-OAll via the side chain.
Standard side chain protecting groups included Gln(Trt), Asn(Trt), Lys(Boc), Orn(Boc),
diaminopropionic acid(Boc), diaminobutyric acid(Boc), His(Trt), Ser(tBu), Trp(Boc), Glu(OtBu). N-methylated amino acids were incorporated either as Fmoc-protected N-methylated monomers or by selective N-methylation of the appropriate position on solid support during the synthesis\(^1\). Chain elongations were achieved using 1,3-diisopropylcarbodiimide (DIC) and HOBt in \(N\)-methylpyrrolidin-2-one (NMP) with 90-min couplings. Fmoc deprotection was achieved using 25% piperidine in NMP. After full elongation of the peptide, the terminal Fmoc was removed, and then the allyl group was cleaved using 0.5 mol-equiv of \(\text{Pd(PPh}_3\text{)}_4\) with 10 mol-equiv of PhSiH\(_3\) in CH\(_2\)Cl\(_2\) (2 x 1.5 h). The peptides were cyclized on resin using bromotripyrrolidino-phosphonium hexafluorophosphate (PyBroP) (5 mol-equiv) and \(\text{i-Pr}_2\text{NEt}\) (12 mol-equiv) in DMF for 2 h. Peptides were cleaved from the resin with concomitant side chain deprotection by agitation in a solution of 95:2:5:2.5 TFA:triisopropylsilane (TIS):water for 3 h. The peptide was precipitated with ether, centrifuged, and washed three additional times with ether. The crude peptides were purified by preparative reverse-phase (RP)-HPLC on a Vydac 218TP C18 or Thermo BioBasic C18 column. Purity was confirmed by analytical RP-HPLC. Purified peptides were characterized by analytical HPLC and LC-mass spectrometry. Analytical RP-HPLC was performed using a Zorbax 300-SB C-18 column connected to a Hitachi D-7000 HPLC system. Binary gradients of solvent A (99% H\(_2\)O, 0.9% acetonitrile, 0.1% TFA) and solvent B (90% acetonitrile, 9.9% H\(_2\)O, 0.07% TFA) were used for HPLC. In cases where peptides were converted from their trifluoroacetate salts to their hydrochloride salts, the peptides were dissolved in 50 mM aqueous HCl and lyophilized for a total of three times, after which \(^{19}\text{F-NMR}\) indicated there was no remaining trifluoroacetate. Characterization data for the peptides used in this study are given in Supplementary Table 3.
**In vitro cecum culture and peptide screening.**

We screened a number of parameters to optimize the *in vitro* culture condition for maximal bacterial diversity in liquid culture, including a survey of several different growth media, dilution factors for plate inoculation, and incubation times; further details of the development of this *in vitro* screen will be reported elsewhere. To prepare peptide stock solutions for the assay, an initial 20 mM peptide stock was made in DMSO, which was diluted to 1 mM in 10% sucrose. From that, a 0.64 mM and 0.16 mM peptide dilution in 10% sucrose was prepared. The use of 10% sucrose in the peptide stock was found to prevent the peptides from precipitating over time in the stock and at once when they were introduced to the inoculated media. The concentration of peptides in stock solutions was calculated based on A280 measurement, using $\varepsilon=5,690 \text{ M}^{-1} \text{ cm}^{-1}$ for Trp and $\varepsilon=1,280 \text{ M}^{-1} \text{ cm}^{-1}$ for Tyr.

Female LDLr−/− mice (8-10 weeks of age) were fed a WD for two weeks. Cecum harvest and all assay steps were performed under anaerobic conditions (Coy Laboratory anaerobic chamber). Cecum contents from three co-housed mice were pooled for the assay to provide enough material and to minimize the individual variation in gut microbiome. Cecum contents were suspended in PBS (1.5 ml PBS per 100 mg of cecum content). The mixture was vortexed for 5 min and left to settle for 5 min. A 1.0 ml aliquot of the supernatant from the settled cecum suspension was removed and centrifuged (2,700 g for 5 min), and the bacterial pellet was flash frozen for use as the non-cultured control. A 10 ml portion of the supernatant from the settled cecum suspension was diluted with 115 mL Chopped Meat Carbohydrate Broth (BD 297307) (1:12.5 dilution) to give a total of 125 ml of inoculated media that was used for *in vitro* peptide screening. We found that Chopped Meat Carbohydrate broth maintained higher bacterial diversity during a 48 h *in vitro* culture compared to other media.
that were examined. The assay was initiated immediately after preparing the inoculated media by mixing 150 µl of a peptide stock (at either 0.64 mM or 0.16 mM) with 1.35 ml of inoculated media in 5 ml snap-cap culture tubes to give 1.5 mL of each assay sample at a final peptide concentration of 64 µM or 16 µM. To monitor the overall bacterial growth profile during the assay, 0.2 ml of each assay sample was removed to a 96 well microplate in a plate reader incubated at 37 °C and OD\textsubscript{600} was measured at 1-hour intervals. The remaining 1.3 mL of each assay sample was incubated at 37°C for 20 h, after which time the bacterial DNA was harvested and isolated with Microbial DNA isolation kit (Mo Bio) following the manufacturer’s instructions. In general, the overall bacterial growth profiles, as monitored by increase in OD\textsubscript{600}, were similar for the peptide-treated samples compared to that of the untreated control sample. In all peptide treatments, the final OD\textsubscript{600} was at least 80% of that observed for the untreated control. Insofar as OD\textsubscript{600} is a proxy for bacterial density, these data support that the overall bacterial density in the peptide-treated conditions remained similar to that of the untreated control.

Following the \textit{in vitro en masse} assay, peptides were scored/ranked as follows. First, we removed peptide sequences that exhibited broad-spectrum antibacterial activity, as defined by causing a $\geq$95% reduction of observed reads compared to the vehicle-treated control for $\geq$20 species. Of the 29 peptides screened, 6 were eliminated due to broad antibacterial activity. The remaining 23 compounds were then ranked based on the degree to which they suppressed the growth of the 19 bacterial species that increased in abundance in WD-fed mice compared to CHD-fed mice (found in Supplementary Table 1). These 19 targeted bacterial species were identified as follows: out of the 54 bacterial genera that showed significant (adjusted p<0.05) changes in abundance in \textit{LDLr}\textsuperscript{-/-} mice fed CHD vs. WD
(Supplementary Table 1), 40 genera decreased while 14 genera increased in abundance with WD feeding. Within the 14 genera that increased in abundance, there were 19 bacterial species that were also present in our *in vitro* culture system. Peptides were scored by summing the observed changes in abundance for each of the identified bacteria, with shifts toward the CHD state being considered positive and shifts away being negative. Compounds scored highly when they reduced the levels of the 19 bacterial species (lowest summed differences in relative abundance). The peptide ranking is found in Supplementary Table 4.

**MIC assays against individual bacterial strains.**

The bacteria used individually in MIC assays were as follows: C. sporogenes (ATCC-15579), C. clostridioforme (ATCC-25537), C. celatum (DSMZ: DSM-1785), P. niger (ATCC-27731), B. acidifaciens (isolated from mouse cecum), P. distasonis (ATCC-8503), P. nigrescens (ATCC-33563), B. breve (strain EX336960VC19 from BEI Resource) and B. longum (strain 44B from BEI Resource). These bacteria were chosen for MIC studies because they represent the two major bacterial phyla found in mouse gut (firmicutes and bacteroidetes), or because they are well-studied probiotics (B. breve and B. longum) from the actinobacteria phylum. Each species was grown in the media recommended by the supplier inside of a vinyl anaerobic chamber (COY Laboratory Products). Each species was characterized using a Bacteria Counting Kit for flow cytometry (LifeTechnologies # B7277) to related OD measurements to CFU/ml for each species. Peptides stocks were prepared in 10% sucrose at 1 mM and frozen in small aliquots at -20°C. Bacterial growth media was inoculated late in the day, one day prior to MIC experiment. On the day of the MIC experiments, peptides were thawed and transferred to the anaerobic chamber. Peptides were diluted 5-fold in the appropriate growth medium corresponding to the given bacteria in a 96-well plate (100 µl, 2x final concentration) and then
serially diluted using growth media by 2-fold into new wells of the plate (50 µl final volume each row, 2x final concentration). Chloramphenicol and ampicillin were used as antibiotic controls. The \( \text{OD}_{650} \) was measured for each bacterial culture and CFU was calculated using the standard curve determined from the bacterial counting kit \( (x = \text{OD}, \ y = \text{CFU/ml}) \). Each species was diluted in its respective growth media to \( 2\times10^5 \) CFU/ml. To initiate the MIC assay, 50 µl of a bacterial culture was added to each of the rows already containing 50 µl of the peptide for final starting concentration of \( 1\times10^5 \) CFU/ml and peptide concentrations ranging from \( 100 \mu M \) to 1.56 µM. Chloramphenicol and ampicillin final concentrations were 32 – 0.5 µg/ml in 2-fold decreases. Plates were incubated for 20 – 24 hours at 37°C after which \( \text{OD}_{600} \) readings were taken as an indicator of bacterial growth. The MIC for a given peptide against a given bacteria was defined as the lowest concentration of peptide that inhibited >90% of growth (0% growth was defined as \( \text{OD}_{600} \) of the no bacteria blank, and 100% growth was defined as \( \text{OD}_{600} \) of the untreated bacterial culture).

**Feces sample collection and DNA extraction.**

The feces samples were freshly collected after 2, 6, and 10 weeks of treatment, snap-frozen, and stored at -80°C. Microbial genomic DNA was isolated with PowerSoil DNA isolation kit by following manufacturer’s instructions (Mo Bio).

**16S rRNA gene sequencing and processing.**

PCR amplicons of the V3-V4 16S rRNA region were amplified and sequenced using an Illumina MiSeq platform. For each sample, duplicate 25 µl PCR reactions were performed, each containing 50 ng of purified DNA, 12.5 µl 2X KAPA HiFi HotStart ReadyMix (Kapa biosystem), and 0.15 µM of each primer designed to amplify the V3-V4 region: forward primer (5’-AATGATACGGCGACCACCGAGATCTACACTCTTTCCCTACACGACG
CTCTTCCGATCTNNNNNNTACGCTACGGGAGGCAGCAG-3’) and reverse primer
(5’-CAAGCAGAAGACGGCATACGAGATNNNNNNNNGTGACTGGA
GTTCAAGACGTGCTTTCAAGATCTnnnnnGGACTACHVGGGTWTCTAAT-3’). A
unique 12-base sequence (N’s) was included for sample identification. We also included
unique molecular identifiers (n’s) to correct PCR duplication artifacts during the library
preparation. Cycling conditions were 98°C for 2 min, followed by 20 cycles of 98°C for 10 s,
60°C for 30 s, and 72°C for 45 s. A condition of 72°C for 5 min was used for the final
elongation step. Replicate PCR samples were pooled, and amplicons were qualified with
Quant-iT dsDNA Assay kit (ThermoFisher). Each sample was combined at an equimolar
amount before DNA purification with Agencourt AMPure XP (Beckman Coulter). The
completed library was sequenced on an Illumina MiSeq platform following the Illumina
recommended procedures, to an average depth of 177,286 ± 33,033 read pairs per sample.

16S rRNA gene sequencing data analysis.
We used custom python scripts to demultiplex sequencing reads based on the second
barcode index and remove PCR duplication artifacts found using unique molecular identifiers
prior to analyzing sequences using the mothur\(^2\) (version 1.36.1). We developed a custom
analysis pipeline based on the MiSeq SOP published by the curators of mothur. Briefly,
contigs were generated by combining forward and reverse paired-end reads using the mothur
make.contigs command. 177,286 ± 33,033 contigs were generated per pair (11,346,311
total). The contigs were filtered based on their length (440-480bp for V3-V4 region), and any
contigs with ambiguous bases were removed. Redundant contigs were then consolidated
from the remaining 9,653,596 contigs, and 1,176,445 unique contigs were then aligned to a
reference database (SILVA non-redundant dataset v119). The contigs that didn’t align
properly based on position of alignment and those with homopolymers of length > 8 were removed before the downstream analysis. We performed a pre-clustering step to cluster reads up to 2 bp apart and removed all chimeric sequences. Finally, we used a k-mer based method to classify the sequences to individual taxonomic groups to get counts per group. Scripts used for calculating the Chao1 index can be found at: https://github.com/bmolparia/species_richness. Furthermore, we performed statistical analysis on the 16S data using DESeq2 to identify significantly altered bacterial genera (adjusted p-value < 0.1) and compare the distribution of various groups using principal-component analysis. Shannon diversity was calculated using the following equation, where $H$ is the Shannon diversity index, $G$ is the number of genera present in each sample, and $p_i$ is the relative abundance of each genera within the sample:

$$H = - \sum_{i=1}^{G} p_i \ln(p_i)$$

**Measurement of plasma cholesterol and liver enzyme activities.**

The mice were bled after an overnight fast after 2 and 10 weeks of treatment. The plasma samples were used to determine lipoprotein profiles and the levels of cholesterol, alanine aminotransferase (ALT), and aspartate aminotransferase (AST). Measurements of plasma levels of cholesterol, ALT, and AST were performed as described previously. Briefly, plasma was separated from whole blood immediately by centrifugation of the blood samples at 5,000 rpm for 10 min at 4°C, and was then stored at -80 °C. Plasma total cholesterol was measured using an enzymatic fluorometric method kit (Amplex® red cholesterol assay kit, No. A12216, Life Technologies) according to the manufacturer’s instructions. Plasma ALT and AST concentrations were measured using Infinity ALT (GPT) and AST (GOT) liquid stable assays, respectively, which are colorimetric kinetic assays (Thermo Scientific). Assays were
performed in accordance with the manufacturer's recommendations, adjusting the reagent volumes (20 μL plasma + 200 μL reagent, 0.69 cm light pathlength of the solution in the well) for analysis of samples in 96-well flat-bottom microplate format.

**Pharmacokinetics.**

These experiments were carried out in part courtesy of Carol S. Ryan, Debra J. Search, Ricardo A. Garcia, and David A. Gordon at Bristol Myers Squibb (Hopewell, NJ, USA). Male Balb/C or C57BL/6 mice (20 g) were maintained on a chow diet. The cyclic peptide c[wlwReQeR] was dissolved in PBS containing 1% sucrose or 95% 50 mM acetate buffer containing 10% sucrose, 5% DMSO (pH=4), and sterile filtered through a 0.22-μm syringe filter before injection. Mice were fasted beginning 12 h before dosing, and continued the fast until after the 8-h time point blood draw was completed. Groups of three mice received a dose of the cyclic peptide via intraperitoneal injection (5 mL/kg for subcutaneous and intraperitoneal, 10 mL/kg for oral gavage). Blood was drawn (30–60 μL) from the retro-orbital sinus into a heparinized capillary tube before dosing (0 min) and at different intervals from 30 min to 8 h after dosing. Plasma was isolated immediately from the whole blood by centrifugation at 5,000 rpm for 10 min at 4°C. Immediately after the plasma was isolated, 20 μL of plasma was acidified with 20 μL of 5% TFA to break peptide-protein interactions, and 40 μL of acetonitrile was then added. After vortexing for 30 s, the mixture was centrifuged at 13,000 rpm for 10 min at 4°C. The resultant supernatant was analyzed by using LC-MS SIM as described below.

**LC-MS SIM quantitation of c[wlwReQeR] concentration in plasma.**

Cyclic peptide concentrations were quantified by using reverse-phase HPLC coupled with mass spectrometry. The electrospray ionization mass spectrometry measurements were
carried out in the positive ionization mode using a single quadrupole mass spectrometer (Hewlett Packard HP 1100 MSD series). 10 or 20 μL of sample were injected through a C8 reverse-phase column (Zorbax 300-SB, 4.6 mm×150 mm, 5 μm) using a flow rate of 1.5 mL/min and binary gradients of solvent A (99% H₂O, 0.1% formic acid, 0.01% TFA) and solvent B (99% acetonitrile, 0.1% formic acid, 0.01% TFA). Mass detection was carried out in the selected ion monitoring (SIM) mode for the positive molecular ion, with the optimized fragmentor and capillary voltages of 180 V and 5 kV, respectively. The selected monitoring mass for cyclic peptide c[wLwReQeR] was 592.8 ([M+2H]²⁺). For quantitative calibration, standard curves were established using mouse EDTA-anticoagulated plasma spiked with various concentrations of the peptide. The calibration curve was established by using linear fitting of the data, with correlation coefficient ≥ 0.98.

**LC-MS SIM quantitation of c[wLwReQeR] concentration in feces.**

Extraction solution (1:1 H₂O/acetonitrile containing 0.1% TFA) was added to dried, weighed fecal samples at a volume of 34 mL/g dry feces. The samples were at 50 °C for 3 h. After sonication, the samples were centrifuged at 14,000 rpm for 10 min, and the supernatant was removed and mixed with an equal volume of extraction solution for LC-MS analysis. Cyclic peptide concentrations were determined by using reverse-phase HPLC coupled with mass spectrometry. The electrospray ionization mass spectrometry measurements were carried out using a single quadrupole mass spectrometer (Hewlett Packard HP 1100 MSD series). Chromatographic separations involved a C8 reverse-phase column (Zorbax 300-SB, 4.6 mm×50 mm, 5 μm) using a flow rate of 0.5 mL/min and binary gradients of solvent A (99% H₂O, 0.1% formic acid) and solvent B (99% acetonitrile, 0.1% formic acid). Mass detection was carried out in the selected ion monitoring (SIM) mode with the optimized fragmentor and
capillary voltages of 175 V and 3.5 kV, respectively. The selected ion monitoring masses were 1181.8 ([M-H]⁻) in the negative ion mode for c[wLwReQeR] and 368.2 ([M+3H]³⁺) in the positive ion mode for c[wLwKhShK]. For quantitative calibration, standard curves were established using fecal samples of untreated mice spiked with various concentrations of the peptide prior to the sonication process. The calibration curves were established by using linear fitting of the data, with correlation coefficient ≥ 0.99.

**In vivo cholesterol absorption assay.**

This experiment was carried out courtesy of Carol S. Ryan, Debra J. Search, Ricardo A. Garcia, and David A. Gordon at Bristol Myers Squibb (Hopewell, NJ, USA). To assess the effects of c[wLwReQeR] on intestinal absorption of cholesterol, male LDLr⁻/⁻ mice (Jackson Laboratories) ranging from 8–10 weeks old were acclimated to single housing in standard solid bottom bedded cages for at least one week at BMS facilities. During the acclimation period, the mice were fed a chow diet *ad libitum* (Harlan 2018). At the start of the treatment period and for the duration of the study, mice were switched to high-fat, high-cholesterol Western diet (Research Diets D12108C) containing 1.25% cholesterol and 20% fat *ad libitum*. The peptide was administered in the drinking water *ad libitum* as a solution with 1% sucrose at 180 μM (~35 mg/kg/day). As a positive control, ezetimibe (3 mg/kg) was administered by once daily oral gavage at 5 mL/kg in a formulation containing 0.1% Tween 80 and 0.75% carboxymethyl cellulose.

Following a 2-wk treatment period, cholesterol absorption was measured using a dual fecal isotope ratio method. Mice were fasted for 4 hours and each animal was administrated 50 μl of radioisotope cocktail composed of the following: 1.0 μCi of [¹⁴C]-cholesterol and 2.0 μCi of μ-[³H]-sitosterol mixed in corn oil as a carrier. Feces were collected for a period of 48
hours following administration of radioisotope cocktail. At least 50 µl of blood was collected at 24 h and 48 h following administration of radioisotope cocktail via retro-orbital bleeding and was centrifuged to allow isolation of plasma. Plasma (~20 µl) was added to 5 ml of Opti-fluor Scintillation cocktail (Packard Bioscience #6013199) and counted on ³H and ¹⁴C channels to evaluate cholesterol efflux. Radioactivity in feces was analyzed as follows: for each animal, feces recovered over the 48 hours was dried overnight at 50 °C and 0.1 gram of dried feces was transferred to a glass tube. The feces were dissolved in 0.7 ml water and vortexed to a paste. The sample was then treated with 1.4 ml of 1 N NaOH in EtOH at 85 °C for 2 hours. Lipids were extracted with 3 ml of petroleum ether, and samples were centrifuged at 600 rpm for 5 minutes. A volume of 2.0 ml of the top layer was transferred to a scintillation vial. The samples were dried under a stream of nitrogen gas, and then reconstituted with 100 µl chloroform/methanol (1:2). Finally, 5 ml of scintillation fluid was added to the vials for scintillation counting.

**Plasma lipoprotein profile.**

Pooled plasma (240 µL total, 30 µL from each, n = 8 fasted mice per group) from the two-week blood draw was used for fast protein liquid chromatography (FPLC) analysis. Lipoproteins were separated by using 3 Superdex 200 10/30 columns connected in series (GE Healthcare). The plasma was centrifuged at 11,000 rpm for 10 min at room temperature to remove particulates (floating material was gently mixed into liquid before removing the supernatant for FPLC injection). 200 µL of the pooled plasma was injected on the system eluted with 10 mM tris-HCl buffer, pH 7.4, containing 1 mM EDTA and 150 mM NaCl; a flow rate of 0.5 ml/min and fraction size of 0.5 mL were used.

**Analysis of atherosclerosis.**
Atherosclerotic lesion severity was assessed in the aortae as previously described\(^5\). Briefly, at euthanasia, animals were perfused with PBS, followed by 4\% formaldehyde (10\% UltraPure EM Grade from Polysciences diluted in PBS, pH 7.2). For *en face* analysis, the entire mouse aorta was dissected from the proximal ascending aorta to the bifurcation of the iliac artery by using a dissecting microscope. Adventitial fat was removed, and the aorta was opened longitudinally, pinned flat onto black dissecting wax, stained with Sudan IV, and photographed at a fixed magnification. The photographs were digitized, and total aortic areas and lesion areas were calculated by using Adobe Photoshop and NIH Image J software. The results were reported as a percentage of the total aortic area that contained lesions.

As a second assessment of atherosclerosis, lesions of the aortic root (heart sinus) were analyzed\(^5\). Utilizing stereological principles, lesion volume was estimated across a fixed distance of the aortic sinus. After 10 min fixation in 4\% paraformaldehyde, hearts were cut at an angle perpendicular to the atria of the heart and embedded in OCT (Tissue-Tek). Frozen hearts were sectioned on a Leica cryostat, with 10-\(\mu\)m sections collected from the beginning of the aortic sinus (defined as when a valve leaflet became visible) to 500 \(\mu\)m below the beginning of the sinus. For hearts cut at an angle that resulted in valve leaflets not appearing in the same section (due to poor section angle), the lagging leaflet was used to determine the 500-\(\mu\)m distance. Sections were collected in duplicate at 50-\(\mu\)m intervals. Sections were stained with oil red O, counterstained with Gill hematoxylin 1 (Fischer Scientific International), photographed, and digitized for lesion analysis. Scoring of valve lesion areas was done for each of the 3 valve cusps individually. Lesion areas found only within the valve cusp were measured. Lesion volume estimation was determined from a 1-in-10 sampling rate; hence, valve cusps spaced at 140 \(\mu\)m were used to determine the lesion volume for a total of four
sections analyzed per valve cusp. Lesion volume was calculated from an integration of the measured cross-sectional areas. Prediction of the coefficient of error (CE) in approximating lesion volume was computed using the Cavalieri estimator derived from a covariogram analysis of an ordered set of estimates of cross-sectional areas. This yielded CE values of less than 10% that were acceptable for a stereological computation of lesion volume.

**Antibiotic-induced gut microbiota depletion study.**

We used a gut microbiota depletion procedure described by Staley et al\(^6\). Female LDLr-/- mice (n = 35), 8-20 weeks of age were randomized twice between 7 cages in groups of 5 mice/cage to equalize the microbiota and even out age spread. Housing units (cage, bedding, and water bottles) were autoclaved over the entire course of the study. Food was irradiated by vendors. Mice were fed standard chow diet (PicoLab Rodent Diet 20, #5053, % Kcal from: protein 24.65%, carbohydrate 62.14 %, fat 13.20 %) prior to the study and then switched to a high fat diet (Envigo #TD.94059, % Kcal from: protein 20.4 %, carbohydrate 42.7 %, fat 36.9 %) on day -21. The antibiotic cocktail was prepared fresh daily one day prior to consumption, stored at 4°C, and replaced daily. The cocktail consisted of three nonabsorbable antibiotics each at 1 mg ml\(^{-1}\) in the drinking water (ertapenem sodium salt, neomycin sulfate, vancomycin hydrochloride). Peptides were dissolved in sterile 1.2% sucrose prior to the study and stored at -20°C and then diluted with 10X PBS to final 1X. PBS was used as vehicle control. Final concentrations of peptide were 180 µM in drinking water (corresponding to a dose of ~30 mg kg\(^{-1}\) day\(^{-1}\)). The timeline of the study is shown in Supplementary Figure 1. Blood and fecal samples were collected at each timepoint and mice were weighed. One cage received no treatment as a control. DNA was isolated from the fecal samples using a DNeasy Powersoil kit (Qiagen). Genomic DNA was measured using a
nanodrop 2000. 5 ng of gDNA was amplified using forward and reverse 16Sv3-4 primers (as described elsewhere). Amplicon concentrations were measured using a Qubit dsDNA HS (High Sensitivity) Assay Kit (Thermo Fisher). Equal amounts of each amplicon were combined into one of two libraries (each library containing either 2 or 3 of the 5 replicates from each treatment group, n = 112 or 114 samples, respectively) and cleaned up using Ampure XP beads (Beckman Coulter). Purity was checked using Bioanalyzer High Sensitivity DNA kit (Agilent) and the libraries were sequenced using an Illumina MiSeq (2x300 kit) using a full flow cell run with 20% added PhiX.

**Bacterial RNA-seq sample preparation and sequencing.**

RNA-seq was carried out using a single sample from each group to generate profiling information that could be validated and confirmed by using qPCR and metabolomics analyses. Total bacterial RNA from feces samples was isolated with ZR Soil/Fecal RNA MicroPrep (Zymo research) following the manufacturer’s instructions. Briefly, the sample was suspended in RNA lysis buffer and lysed by the mixer (Retsch). The supernatant was transferred to a RNA-binding column and washed several times with RNA wash buffer. In-column DNaseI digestion at 25°C for 15 min was performed to eliminate DNA contamination in the sample. The presence of genomic DNA contamination was assessed by PCR with universal 16S rRNA gene primers. Before RNA-seq library preparation, rRNA was removed from 2 µg of total bacterial RNA with Ribo-Zero Bacteria kit (Illumina). 100 ng of purified RNA was used for RNA-seq library preparation as described previously. The cDNA was synthesized by reverse transcription with SuperScript III (Life technologies) and second-strand synthesis (New England Biolabs). The sequencing library was generated from purified cDNA with Nextera XT DNA library preparation kit (Illumina) and amplified by PCR. Cycling
conditions were 72°C for 3 min, 95°C for 30 s, followed by 16 cycles of 95°C for 10 s, 55°C for 30 s, and 72°C for 30 s. A condition of 72°C for 5 min was used for the final elongation step. Libraries with different indexes were pooled and sequenced on an Illumina NextSeq at the Scripps Research Institute next generation sequencing core. 4,453,883, 8,956,883, and 9,166,765 reads were generated from the CHD, WD, and WD + c[wLwReQeR] samples, respectively. Of these reads, 1,367,533 were mappable for CHD (30.7% of total generated reads), 4,411,184, were mappable for WD (49.2% of total generated reads), and 4,470,591 were mappable WD + c[wLwReQeR] (48.8% of total generated reads).

**Functional analysis of bacterial RNA-seq data.**

We used BWA (version 0.7.12) read alignment program\(^8\) and a custom-built bacterial reference database, which contains 4,700 bacterial genomes from NCBI NR sequence database, to map RNA-seq reads. The reads aligned to individual bacterial genes were counted using HTSeq count\(^9\) (version 0.6.0) and the counts were further normalized based on size factors estimated using the median of the ratios of observed counts method as described previously\(^10\). The proteins predicted by RNA-seq read alignment were annotated with DIAMOND\(^11\) (version 0.8.26) using the NCBI nonredundant (NCBI-nr) database\(^12\) and the default setting (BLASTX e value < 10\(^{-3}\), bit score > 50). For query genes with multiple matches, the annotated reference gene with the lowest e value was chosen. The functional analysis was performed by MEGAN\(^13\) (version 5.11.3) and KEGG analyzer was used to determine read counts in different metabolic pathways from each group. The aggregate expression level changes for sequences within a given function were normalized before the comparison between each group.

**SCFA and amino acid measurements.**
Fecal SCFA and amino acid content was measured by liquid chromatography-mass spectrometry (LC-MS). The fecal samples were weighed and placed into 2 ml Omni tubes. Ethanol was added to bring the volume to 350 µl. Next, 4 µl of 500 µM deuterated FA mix and 20 µl of 1 mM 3-Cyclohexanepropionic acid (3CHPA) were added to each sample as internal standard. The samples were homogenized using an Omni bead beater at the speed of 4.5 m/s for 30 sec, and then centrifuged at 13,000 rpm for 15 min. 90 µl of the supernatants was transferred into glass HPLC vials. 30 µl of freshly prepared 4X 3NPH solution (160 mM 3-nitrophenylhydrazine, 150 mM EDC, pyridine 12% vol, 50% acetonitrile) was added to each sample and the reactions were held at room temperature for 30 min. 2 µl of the sample was injected into the LC-MS (Agilent 1290 HPLC, 6550 qTOF) with a Kinetex C18 reversed phase column (2.6 µm, 150 x 2.1 mm i.d., Phenomenex). The following LC solvents were used: solution A, 0.1% formic acid in water, solution B, acetonitrile containing 0.1% formic acid. The following LC gradient was applied: at a flow rate of 0.4 mL/min, 15% B at 0 min and ramp up B% to 40% in 8.5 min, then ramping to 70% B at 10 min, followed by 99% B at 14 min, maintaining at 99% B for 2 min and reduce to 15% B for 0.5 min and re-equilibrated to 15% B for 3.5 min, for a total run time of 20 min. The concentration of each SCFA and amino acid was calculated based on a standard curve and normalized by the internal control and the feces weight.

**Mouse RNA-seq sample preparation and sequencing.**

Mouse tissues were harvested after 2 weeks of treatment, stored in RNAlater, and snap-frozen. 20-30 mg of mouse tissue was used for RNA extraction with RNeasy Mini Kit (Qiagen) following the manufacturer’s instructions. Ribosomal RNA was removed from total RNA with Ribo-Zero Gold rRNA removal kit (Illumina). Strand-specific RNA-seq libraries were
constructed as described previously\textsuperscript{14}. Libraries with different barcodes were pooled and single-end sequencing (75bp) was performed on an Illumina NextSeq at the Scripps Research Institute next generation sequencing core.

**Mouse RNA sequencing data analysis.**

After ribosomal RNA sequences were removed, the RNA transcripts were quantified using RSEM\textsuperscript{15}. Differentially expressed genes were identified by DESeq\textsuperscript{23} and significantly changed genes were selected using a cutoff of adjusted p-value < 0.1, comparing peptide-treated animals (n=3) to vehicle-control animals (n=3) for each group (CHD or WD-fed animals). Enrichment of Gene Ontology terms and categories was performed with DAVID 6.8\textsuperscript{16,17}.

**Quantitative real-time PCR.**

To validate selected gene expression results, qPCR reactions were carried out. Mouse livers were harvested two weeks after peptide treatment and total RNA was extracted using RNeasy Mini (Qiagen) following manufacturer’s instructions. Reverse transcription was performed for 1 h using random priming (Promega). qPCR reactions (0.5 µl cDNA, 0.2 µM each primer, SYBR green Master Mix (Kapa biosystems)) were performed on a Bio-Rad CFX384 Touch Real-Time PCR detection system, using primers specific for each gene (Supplementary Table 8). Data were normalized to loading controls (16S and β-actin).

**Bulk bile acid quantification.**

Total bile acids were measured enzymatically using a total bile acid assay kit (Cell Biolabs) following manufacturer’s instruction. Feces were obtained between 0900-1100 am from non-fasted animals after a 2-wk treatment period. The extraction of bile acid from feces was performed as described previously\textsuperscript{18}. In brief, the feces from individual mice were collected,
weighed, and agitated in 75% ethanol in an ultrasonic bath at 50°C for 2 h. After centrifugation at 3,500 g for 10 min, the supernatant was transferred to a new tube and diluted with 25% PBS. The total bile acid level was then determined enzymatically using the kit. For plasma samples, the plasma was diluted 1:10 with 25% PBS prior to enzymatic bile acid measurement using the kit.

**Measurement of individual bile acids.**

Feces were obtained between 0900-1100 am from non-fasted animals after a 2-wk treatment period. Bile acids were extracted from samples according to published procedures. Briefly, fecal samples were lyophilized and homogenized. Five milligrams of fecal powder were extracted with 250 µL of methanol containing heavy internal standards. Fifty microliters of plasma were extracted with 150 µL of methanol containing heavy internal standards. After vortexing for 10 minutes and centrifuging (16,000 x g, 4 C, 10 min), supernatants were transferred to glass vials for injection and analysis by LCMS. Bile acids were analyzed on a Dionex Ultimate 3000 LC system (Thermo) coupled to a TSQ Quantiva mass spectrometer (Thermo) fitted with a Kinetex C18 reversed phase column (2.6 µm, 150 x 2.1 mm i.d., Phenomenex). The following LC solvents were used: solution A, 0.1% formic acid and 20 mM ammonium acetate in water, solution B, acetonitrile/methanol (3/1, v/v) containing 0.1% formic acid and 20 mM ammonium acetate. The following reversed phase gradient was utilized: at a flow rate of 0.2 mL/min with a gradient consisting of 25-29% B in 1 min, 29-33% B in 14 min, 33-70% B in 15 min, up to 100% B in 1 min, 100% B for 9 min and re-equilibrated to 25% B for 10 min, for a total run time of 50 min. The injection volume for all samples was 10 µL, the column oven temperature was set to 50 °C and the autosampler kept at 4 °C. MS analyses were performed using electrospray ionization in positive and negative
ion modes, with spray voltages of 3.5 and -3 kV, respectively, ion transfer tube temperature of 325 °C, and vaporizer temperature of 275 °C. Multiple reaction monitoring (MRM) was performed by using mass transitions between specific parent ions into corresponding fragment ions for each analyte.

**Quantification of regulatory T cells.**

Lamina propria (LP) immune cells from the small intestine were isolated using lamina propria dissociation kit (Miltenyi Biotec) following the manufacturer’s instruction. All antibodies and flow cytometry reagents were obtained from Thermo Fisher. We used FITC anti-CD4 (RM4-5), PE anti-Foxp3 (FJK-16s), PE-Cyanine 7 anti-GATA3 (TWAJ), APC anti ROR gamma t (AFKJS-9), APC-efluor780 anti-Helios (22F6) and e-fluor 450 anti-CD3 (17A2) antibodies. To block non-antigen specific binding of immunoglobulins and discriminate live from dead cells, LP cell suspensions were incubated with mouse BD Fc block (BD Biosciences) and e-fluor 506 fixable viability dye (Thermo Fisher) for 30 minutes on ice. For surface staining, the cells were incubated with antibodies for 25 min, washed and fixed in fixation/permeabilization solution (Thermo Fisher). After being washed with transcription factor wash buffer (Thermo Fisher), cells were stained intracellularly for 45 min at room temperature, washed, and resuspended in PBS. Samples were read in a FACs canto analyzer (BD Biosciences) and analyzed with FACs Diva software v 8.0 (BD Biosciences). Total LP cell numbers were quantified to calculate regulatory T cell ratios. Flow Jo v10 software was used for dot plots generation and final analysis.

**Statistical analysis.**

Data are expressed as the mean ± S.D. Statistical significance was determined by analysis of variance (ANOVA) with Tukey post hoc test, or Student’s t-test, as determined by using
GraphPad Prism software (version 8.3.1). p values <0.05 were considered as statistically significant for *in vivo* animal study.

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Supplementary Figures

Supplementary Figure 1. Peptide mediated effects on cholesterol and triglyceride levels. a, Administration of the peptides (~35 mg/kg daily oral dosing for 2-wk) reduced plasma total cholesterol in WD-fed LDLr⁻/⁻ mice compared to the WD fed vehicle group (n=4 per group). CHD-fed mice had lower plasma total cholesterol levels and no effects were observed on these levels by peptide treatment. Scatter plots are shown with mean ± SD. p values were determined by two-tailed Student's t-test. b, Administration of the peptides (~35 mg/kg daily oral dosing for 2-wk) reduced plasma total cholesterol in WD-fed LDLr⁻/⁻ mice compared to the WD fed vehicle group (n=18 animals for vehicle group; n=9 animals for c[wLwReQeR] group; n=8 animals each for the other groups). Scatter plots are shown with mean ± SD. The p values were determined by one-way ANOVA. These are the raw data for the ‘intact microbiota’ study shown in Fig. 4a of the main text. c, Plasma total cholesterol levels after a 10-wk treatment period (n=18 animals for vehicle group; n=8 animals each for the other groups). Scatter plots are shown with mean ± SD. The p values were determined by one-way ANOVA. (d-g) In the fecal dual isotope cholesterol absorption assay, each animal (n=8/group) received c[wLwReQeR], ezetimibe (a positive control inhibitor of cholesterol absorption), or vehicle controls orally for 2 weeks, after which they were dosed with a mixture of ¹⁴C-cholesterol and ³H-sitosterol radiolabeled sterols. Feces were collected over a period of 48 h, and plasma was taken at 24 h and 48 h post dosing of the radiolabeled sterols. Animals were fed WD over the full course of the study. Peptide c[wLwReQeR] was administered at ~35 mg/kg in 1% sucrose/PBS as the drinking water ad libitum. Ezetimide was administered by daily oral gavage (3-mg/kg dose) formulated in 0.1% Tween 80/0.75% carboxymethylcellulose. d, Plasma levels of ¹⁴C-cholesterol measured from blood drawn at 24 and 48 h after sterol dosing. In contrast to ezetimibe, c[wLwReQeR] did not reduce absorption of dietary cholesterol in vivo. e, Levels of ¹⁴C-cholesterol measured from feces collected over the 48 h after sterol dosing. Ezetimide increased excretion of dietary cholesterol by 87%, whereas c[wLwReQeR] did not affect cholesterol excretion. f, Plasma total cholesterol levels were reduced by both treatment compounds. g, Plasma triglyceride levels were significantly reduced by c[wLwReQeR], but not by ezetimibe. All charts are shown as mean ± SD. In panels d-g, statistical significance was determined by two-sided Student's t-tests comparing the treatment groups with their corresponding vehicle group.
Supplementary Figure 2. Gut microbiota are required for peptide *in vivo* activity, as indicated by using antibiotic-induced gut microbiota depleted mice. 

**a**. Dosing schedule for the study. **b**, Oral administration of 11 did not significantly reduce total plasma cholesterol compared to vehicle in gut microbiota-depleted LDLr-/- mice with continuing antibiotic treatment, but 11 did cause a statistically significant reduction in plasma cholesterol when antibiotic treatment was discontinued (adjusted p-values were determined by one-way ANOVA with post-hoc Dunnett test). These findings are consistent with the gut microbiota being required for the phenotypic effects observed for 11. All groups contained n=5 mice. Scatter plots are shown with mean ± SD. The 'continued antibiotics' data are the raw data for the depleted microbiota study shown in Fig. 4a of the main text. 'ns', not significant. **c**, Plot of microbial DNA levels obtained per fecal pellet following the antibiotic depletion. Compared to samples taken before administration of the antibiotic cocktail or animals that did not receive antibiotic, bacterial DNA levels were significantly reduced in the continuing antibiotic-treated animals, indicating that the gut microbiota was substantially depleted in the study. In the animals for which antibiotic treatment was discontinued, the quantities of recovered microbial DNA increased over the course of 14 days to return to a similar level as that of
untreated animals (fecal samples were taken from the following numbers of individual animals for each condition: n=45 animals for pre-antibiotic group; n=5 animals for no treatment groups; n=40 animals in day 0 antibiotic group; n=19 animals in day 7 continued antibiotic group; n=20 animals in day 7 discontinued antibiotic group; n=19 animals in day 14 continued antibiotic group; n=18 animals in day 14 discontinued antibiotic group.). Microbial genomic DNA was isolated using a PowerSoil DNA isolation kit by following manufacturer’s instructions (Mo Bio). Data are shown as scatter plots with mean ± SD, and adjusted p-values were determined by one-way ANOVA with post-hoc Tukey test. d, Plot of Shannon diversity (genera) of microbiota samples over the course of the antibiotic-induced microbiome depletion study. A significant reduction in alpha diversity was observed following antibiotic treatment, as can be seen by comparing diversity values for each group on day -7 to day 0 (single-tailed paired Student’s t-test with unequal variance, p=1E-7; n=5 animals for all groups, except n=4 animals for the continued antibiotic + c[wMeLwRMeeQeR] group on day 7 and day 14). In the box plot, lower and upper box boundaries correspond to 25th and 75th percentiles, respectively; the line inside box is the median; the lower and upper error lines are the minimum and maximum values, respectively; and the dots are outliers (more than 1.5x outside the interquartile range). As would be expected, mice from cages for which antibiotic was discontinued saw a more rapid return in biodiversity compared with continuing antibiotic groups. No cage saw biodiversity return completely to pre-antibiotic levels by day 14 (single-tailed paired Student’s t-test, p=5E-5). Despite the general observed increase in biodiversity, the relative abundances of genera were not necessarily similar to their pre-antibiotic state. e, Bar graphs showing mean genera relative abundance (mean of n=5 animals/group) over the course of the study. Antibiotic treatment caused a dramatic shift in genera abundance, characterized by an increase of Lactococcus and Mycoplasma, which were present in minute quantities before antibiotic treatment. All other genera were greatly reduced in relative abundance. At day 7, animals with discontinued antibiotic treatment were regaining a microbiota community. Analysis of day 14 data revealed that the antibiotic treatment further maintained the antibiotic-depleted population dominated by Lactococcus and Mycoplasma but that other genera (Flavobacterium in the Bacteroidetes phyla and several genera in the Proteobacteria phyla) were increasing in relative abundance. Among the discontinued antibiotic groups, all had lost the relative dominance Lactococcus and Mycoplasma genera that characterized the antibiotic treatment and were regaining the Lachnosperaceae-unclassified genera (Firmicutes phyla) and Porphyromonadaceae-unclassified genera (Bacteroidetes phyla).
Supplementary Figure 3. Observed changes of inflammation- and tight junction related genes and morphology mediated by c[wLwReQeR].

(a) RNA-Seq analysis of inflammation-related genes in LDLr\(^{-/-}\) mice liver tissue. In general, the genes were downregulated by peptide treatment. Among these genes are several proinflammatory cytokines that are upregulated by WD-feeding and restored to lower levels by peptide treatment, suggesting that peptide treatment exerts anti-inflammatory effects.

(b) Heatmap showing expression changes for selected genes related to tight junction function in ileum after a 2-wk treatment period. Peptide treatment upregulated several of the genes, which would reduce leakage from the intestine and thereby reduce inflammation.

(c) Quantification of length of villi + crypt in ileum from CHD, WD, or peptide-treated animals (villi length was determined from a total of 68, 90, and 76 individual ileum measurements taken from n=7, 8, and 5 animals for the CHD, WD+vehicle, and WD+peptide groups, respectively). Data are shown as scatter plots with mean ± SD. Adjusted \(p\)-values were determined by one-way ANOVA with post-hoc Tukey test.

(d) Heat-map showing expression levels in genes for which there were significant differences (significance determined by using modified Fisher Exact \(p\)-value for gene-enrichment analysis) between the peptide-treated WD and vehicle WD groups, as determined by RNA-Seq. Samples were from ileum (small intestine) tissue after a 2-wk treatment period. Four total groups of animals were analyzed: two WD and two CHD groups treated either with peptide or vehicle. The altered genes were grouped into two clusters for which peptide treatment caused increases (Cluster 3) or decreases (Cluster 4) in expression level.

(e) Gene ontology analysis for genes that were significantly changed in ileum (small intestine) tissue, when comparing peptide-treated WD group to vehicle-treated WD group (n=3 animals per group).
Supplementary Figure 4. Absolute cell counts and gating strategy used in Treg analyses. n =3 mice each for peptide-treated WT and LDLr⁻/- groups; n=4 animals for vehicle-treated WT group; n=5 animals for vehicle-treated LDLr⁻/- group. a, Representative flow cytometry plots and graphs of cell counts for each condition, shown as mean ± SD. p values were determined by two-tailed Student’s t-test. b, Gating strategy used for analysis of RORγt⁺Foxp3⁺ Tregs, Helios⁺Foxp3⁺ Tregs, and Th17 cells in lamina propria of small intestine.
### Supplementary Tables

**Supplementary Table 1.** List of bacterial taxa in cecum samples of LDLr<sup>−/−</sup> mice (n=8 mice per group) for which abundance significantly changed between CHD feeding and WD feeding.

| taxon | Fold change (WD/CHD) | taxon | Fold change (WD/CHD) |
|-------|----------------------|-------|----------------------|
| g_Anaeroplasma | 0.003 | g_Parasutterella | 1.77 |
| f_Erysipelotrichaceae, g_Incertae_Sedis | 0.012 | g_Desulfovibrio | 1.79 |
| p_Firmicutes, c_unclassified | 0.04 | g_Bacteroides | 2.75 |
| o_RF9, f_unclassified | 0.05 | g_Butyricicoccus | 3.51 |
| g_Anaerovorax | 0.06 | g_Lactococcus | 3.65 |
| g_Dorea | 0.08 | g_Allobaculum | 4.19 |
| f_Erysipelotrichaceae, g_unclassified | 0.13 | g_Parabacteroides | 4.24 |
| g_Rikenella | 0.13 | p_Bacteroidetes, c_unclassified | 6.00 |
| f_Rikenellaceae, g_unclassified | 0.14 | f_Prevotellaceae, g_unclassified | 7.63 |
| g_Papillibacter | 0.14 | g_Bifidobacterium | 19.18 |
| c_Clostridia, o_unclassified | 0.17 | g_Alloprevotella | 97.03 |
| g_Flavonifractor | 0.18 | g_Rhizobium | not obs in CHD |
| f_vadinBB60, g_unclassified | 0.19 | g_Candidatus_Arthromitus | not obs in CHD |
| f_Family_XIII, g_unclassified | 0.19 | f_Rhizobiaceae, g_unclassified | not obs in CHD |
| g_Anaerofustis | 0.19 |
| f_Ruminococcaceae,g_Incertae_Sedis | 0.21 |
| g_Oscillibacter | 0.23 |
| g_Roseburia | 0.23 |
| g_Gordonibacter | 0.24 |
| p_Proteobacteria, c_unclassified | 0.24 |
| g_Streptococcus | 0.26 |
| g_Odoribacter | 0.27 |
| g_Intestinimonas | 0.29 |
| g_Candidatus_Saccharimonas | 0.31 |
| g_Enterorhabdus | 0.31 |
| g_Anaerotrunucus | 0.32 |
| f_Defluviitaleaceae,g_Incertae_Sedis | 0.32 |
| o_Lactobacillales, f_unclassified | 0.32 |
| c_Betaproteobacteria, o_unclassified | 0.34 |
| g_Coprococcus | 0.35 |
| f_Ruminococcaceae, g_unclassified | 0.38 |
| f_Coriobacteriaceae, g_unclassified | 0.39 |
| g_RC9_gut_group | 0.42 |
| g_Oscillibacter | 0.44 |
| o_Clostridiales, f_unclassified | 0.48 |
| g_Acetatifactor | 0.50 |
| g_Lactobacillus | 0.51 |
| f_Desulfovibrionaceae, g_unclassified | 0.53 |
| f_Lachnospiraceae, g_unclassified | 0.53 |
| f_Family_XIII, g_Incertae_Sedis | 0.59 |

Significance was defined as adjusted p<0.1, as determined by DESeq2 using a two-sided Wald test with adjustment for multiple comparisons using the Benjamini-Hochberg method).
Supplementary Table 2. List of bacterial taxa observed in uncultured cecum samples of LDLr-/- mice and in the in vitro en masse screening assay.

| taxon                                      | Present within in vitro culture? | taxon                                      | Present within in vitro culture? |
|--------------------------------------------|----------------------------------|--------------------------------------------|----------------------------------|
| g_Candidatus_Saccharimonas                 | -                                | o_Bacteroidales, f_unclassified            | +                                |
| g_Staphylococcus                           | -                                | p_Bacteroidetes, c_unclassified            | +                                |
| g_Lactococcus                              | -                                | o_Gastranaerophilales, f_unclassified      | +                                |
| g_Anaerofustis                             | -                                | g_Mucispirillum                           | +                                |
| f_Family_XIII, g_unclassified              | -                                | g_Lactobacillus                           | +                                |
| possible_genus_Sk018                       | -                                | g_Streptococcus                           | +                                |
| g_Peptococcus                              | -                                | o_Lactobacillales, f_unclassified          | +                                |
| g_Oscillospira                             | -                                | o_Lactobacillales, f_Incertae_Sedis       | +                                |
| g_Pseudoflavonifractor                     | -                                | f_Family_XIII, g_Incertae_Sedis           | +                                |
| g_Sporobacter                              | -                                | f_vadinBB60, g_unclassified               | +                                |
| f_vadinBB60, g_unclassified                | -                                | g_Acetatifactor                           | +                                |
| c_Clostridia, o_unclassified               | -                                | g_Blautia                                 | +                                |
| g_Thalassospira                            | -                                | g_Coprococcus                             | +                                |
| g_Bilophila                                | -                                | f_Lachnospiraceae, g_Incertae_Sedis       | +                                |
| f_Desulfovibronaceae, g_unclassified       | -                                | g_Marvinbryantia                          | +                                |
| g_Ureaplasma                               | -                                | g_Roseburia                               | +                                |
| g_Akermansia                               | +                                | f_Lachnospiraceae, g_unclassified          | +                                |
| g_Bifidobacterium                          | +                                | g_Dehalobacterium                         | +                                |
| g_Enterorhabdus                            | +                                | f_Peptostreptococcaceae, g_Incertae_Sedis | +                                |
| g_Olsenella                                | +                                | g_Aeroerotrunccus                         | +                                |
| g_Parvibacter                              | +                                | f_Ruminococcaceae, g_Incertae_Sedis       | +                                |
| f_Coriobacteriaceae, g_unclassified        | +                                | g_Intestinimonas                          | +                                |
| g_Bacteroides                              | +                                | g_Oscillibacter                           | +                                |
| g_Odoribacter                              | +                                | f_Ruminococcaceae, g_unclassified          | +                                |
| g_Parabacteroides                          | +                                | o_Clostridiales, f_unclassified           | +                                |
| g_Aloprevotella                            | +                                | g_Alobaculum                              | +                                |
| g_Prevotella                               | +                                | f_Erysipelotrichaceae, g_unclassified     | +                                |
| f_Prevotellaceae, g_unclassified           | +                                | p_Firmicutes, c_unclassified              | +                                |
| g_Alistipes                                | +                                | g_Parasutterella                          | +                                |
| g_RC9_gut_group                            | +                                | c_Betaproteobacteria, o_unclassified      | +                                |
| g_Rikenella                                | +                                | g_Desulfovibrio                           | +                                |
| f_Rikenellaceae, g_unclassified            | +                                | g_Helicobacter                            | +                                |
| f_S24-7, g_unclassified                    | +                                | p_unclassified                            | +                                |
Supplementary Table 3. HPLC and mass spectrometric characterization data for peptides used in the study.

| sequence       | peptide # | formula (M+H) | expected mass (M+H) | observed mass (M+H) | HPLC purity |
|----------------|-----------|---------------|---------------------|---------------------|-------------|
| wLWHsqK       | 1         | C56H72N16O9   | 1103.5903           | 1103.5931           | >98         |
| wLFkwKK       | 2         | C61H80N14O8   | 1145.6988           | 1145.6968           | >98         |
| wFKKsKsKs     | 3         | C47H71N12O11  | 979.5365            | 979.5375            | >90         |
| WlWlHssKk     | 4         | C55H69N14O9   | 1079.6154           | 1079.6169           | >90         |
| WlWlKkKs      | 5         | C55H68N13O9   | 1070.6515           | 1070.6519           | >95         |
| LiWhKk        | 6         | C60H69N12O7   | 806.4677            | 806.4673            | >98         |
| WlLlKksKs     | 7         | C50H68N12O9   | 997.6562            | 997.6562            | >90         |
| WlWkksKs      | 8         | C37H72N10O10  | 1117.5947           | 1117.5924           | >95         |
| WlWlWkSk      | 9         | C60H69N13O9   | 1128.6358           | 1128.6323           | >98         |
| lflAIkkK      | 10        | C48H69N12O8   | 951.6144            | 951.6141            | >95         |
| WlWkReQer     | 11        | C55H70N12O13  | 1184.5965           | 1184.5936           | >98         |
| FwHyYoHq      | 12        | C57H72N15O10  | 1126.5587           | 1126.5566           | >90         |
| YlWyKhae      | 13        | C55H70N12O12  | 1090.5236           | 1090.5464           | >95         |
| YlYlYsSo      | 14        | C33H72N10O12  | 1045.5722           | 1045.5726           | >95         |
| fnWqHyQ       | 15        | C62H69N10O11  | 1213.5332           | 1213.5309           | >95         |
| WlEwLwksK     | 16        | C54H72N12O11  | 1071.5991           | 1071.5962           | >98         |
| WlWkKrK       | 17        | C58H69N17O8   | 1154.7315           | 1154.7297           | >90         |
| WlWlKrKh      | 18        | C68H80N17O8   | 1148.6845           | 1148.6808           | >90         |
| WlWlKrKr      | 19        | C58H80N12O3   | 1167.7267           | 1167.7250           | >95         |
| WlWwKkKk      | 20        | C63H80N15O8   | 1184.7097           | 1184.7068           | >95         |
| WlHzWkKr      | 21        | C58H80N17O8   | 1148.6845           | 1148.6838           | >98         |
| WlYkKk        | 22        | C54H70N10O7   | 847.5194            | 847.5191            | >95         |
| fWvYKk        | 23        | C46H69N6O7    | 852.4772            | 852.4758            | >98         |
| lWHzOk        | 24        | C40H69N12O6   | 792.4885            | 792.4881            | >98         |
| WlWwEhKk      | 25        | C53H71N14O12  | 1095.5376           | 1096.5299           | >95         |
| YwElYsKq      | 26        | C54H72N14O14  | 1098.5263           | 1098.524            | >98         |
| WqOqtdKt      | 27        | C52H70N12O12  | 1096.5328           | 1096.5305           | >98         |
| WlWwEhSo      | 28        | C49H69N12O13  | 1031.4951           | 1031.4952           | >90         |
| WlLlEeKkNn    | 29        | C40H70N12O13  | 1041.5733           | 1041.5721           | >95         |
| W(***l)wR(***e)QeR | 30 | C57H69N17O13  | 1212.6278           | 1213.6166           | >95         |
| WlWnReQeR     | 31        | C55H70N17O13  | 1184.5965           | 1184.5940           | >98         |
| WlWREQeR      | 32        | C55H70N17O13  | 1184.5965           | 1184.5930           | >98         |
| WlW(***w)W(***e)QeR | 33 | C57H69N17O13  | 1212.6278           | 1212.6233           | >95         |
| WlWnReQeR     | 34        | C55H70N17O13  | 1184.5965           | 1184.5946           | >95         |
| WlWkKsHK      | 35        | C56H72N16O9   | 1103.5903           | 1103.5886           | >98         |
| WlWkKsHK      | 36        | C56H72N16O9   | 1103.5903           | 1103.5887           | >95         |

- Peptide sequences are shown with L-amino acids as upper-case letters and D-amino acids as lower-case letters, using standard one-letter amino acid codes. O, ornithine. HPLC purity was determined by integrating product and impurity peaks monitored at 280 nm or 230 nm. Peptide masses were determined in the Scripps Research Automated Synthesis core facility using a Waters I-Class LC with diode array and G2-XS time of flight (TOF) mass detector.
Supplementary Table 4. Ranking of peptides in remodeling WD gut microbiota toward CHD microbiota composition determined by the in vitro en masse screening assay.

| sequence         | peptide # | peptide activity group | ranked distance to chow |
|------------------|-----------|------------------------|-------------------------|
| WlWlWkSk         | 9         | III                    | 1                       |
| wLwKhShK         | 1         | I                      | 2                       |
| WwLlHsKk         | 4         | II                     | 3                       |
| Chm- 2 µg        | N/A       | V                      | 4                       |
| LlWhQk           | 6         | III                    | 5                       |
| wLfKwKkK         | 2         | I                      | 6                       |
| lLwHoK           | 24        | V                      | 7                       |
| lFlAlKhK         | 10        | III                    | 8                       |
| wLeLwKsK         | 16        | III                    | 9                       |
| wLlWkKkS         | 5         | III                    | 10                      |
| wLhLwKrK         | 21        | IV                     | 11                      |
| wWwKsKsK         | 8         | III                    | 12                      |
| YlYlYkSo         | 14        | III                    | 13                      |
| wLwReQeR         | 11        | III                    | 14                      |
| WwQoHdKt         | 27        | VI                     | 15                      |
| wLwSeQsO         | 28        | VI                     | 16                      |
| YwElYsKq         | 26        | VI                     | 17                      |
| FwHlYoHq         | 12        | III                    | 18                      |
| wFkSkSkS         | 3         | I                      | 19                      |
| WlLlKkKs         | 7         | III                    | 20                      |
| wLlEeKkN         | 29        | VI                     | 21                      |
| wLwSeQhK         | 25        | VI                     | 22                      |
| YlWyKhAe         | 13        | III                    | 23                      |
| Chm- 8 µg        | N/A       | V                      | 24                      |
| fWwYqHhQ         | 15        | III                    | 25                      |

“Peptide activity group” refers to the six groups of peptides observed in a pairwise comparison of peptide activity in remodeling gut microbiota composition shown in Figure 1d. Upper case letters refer to L-amino acids, lower case letters refer to D-amino acids. Standard one letter amino acid abbreviations are used. O, ornithine; Chm, chloramphenicol.
Supplementary Table 5. Pharmacokinetic data for c[wLwReQeR] via different routes of administration.

| Route | intraperitoneal | intraperitoneal | subcutaneous | oral gavage |
|-------|----------------|----------------|--------------|-------------|
| Vehicle | 95% 50 mM acetate buffer containing 10% sucrose, 5% DMSO, pH=4 | phosphate-buffered saline containing 10% sucrose | 95% 50 mM acetate buffer containing 10% sucrose, 5% DMSO, pH=4 | 95% 50 mM acetate buffer containing 10% sucrose, 5% DMSO, pH=4 |
| Mouse strain | C57Bl/6 | Balb/C | C57Bl/6 | C57Bl/6 |
| Dose (mg/kg) | 7.5 | 16 | 7.5 | 15 |
| Cmax (nM) | 1,133 | 12,800 | 530 | <3 |
| Tmax (h) | 0.5 | 0.5 | 0.5 | <3 |
| AUC 0-24h (nM h) | 3,469 | 51,751 | 1,810 | <3 |

Cmax = maximum observed concentration, Tmax = timepoint at which maximum concentration was observed. n=3 animals per timepoint. The lower limit of quantitation was 3 nM.
Supplementary Table 6. Observed levels of bile acids in feces and plasma of LDLr-/- mice following 2-wk of treatment, as determined by targeted metabolomics.

| bile acid     | CHD     | WD + vehicle | WD + c[wLwReQeR] | classification |
|--------------|---------|--------------|------------------|----------------|
|              | Feces   |              |                  |                |
| CA           | 2300 ± 3000 | 640 ± 570 | 440 ± 490 | primary |
| α-MCA        | 20 ± 30 | 38 ± 13 | 21 ± 8.9 | primary |
| β-MCA        | 34 ± 22 | 110 ± 30 | 65 ± 12 | primary |
| ω-MCA        | 160 ± 91 | 240 ± 63 | 170 ± 100 | primary |
| HCA          | 0.82 ± 0.39 | 2.9 ± 1.1 | 1.6 ± 1.1 | primary |
| LCA          | 160 ± 65 | 560 ± 280 | 400 ± 170 | secondary |
| UDCA         | 24 ± 14 | 79 ± 29 | 43 ± 16 | secondary |
| DCA          | 4800 ± 1700 | 5000 ± 820 | 4900 ± 2400 | secondary |
| T-CA         | 15 ± 11 | 4.1 ± 1.1 | 8.3 ± 3.3 | conjugated primary |
| T-α-MCA      | 3.8 ± 3.2 | 2.8 ± 0.61 | 17 ± 8.7 | conjugated primary |
| T-β-MCA      | 4.3 ± 3.6 | 3.2 ± 0.71 | 20 ± 11 | conjugated primary |
| T-ω-MCA      | 2.6 ± 2.2 | 0.95 ± 0.21 | 3 ± 1.4 | conjugated primary |
| T-HCA        | 0.080 ± 0.03 | 0.078 ± 0.02 | 0.097 ± 0.03 | conjugated primary |
| T-DCA        | 6.3 ± 7.5 | 0.67 ± 0.18 | 2.6 ± 1.2 | conjugated secondary |
|              | Plasma  |              |                  |                |
| CA           | 12000 ± 13000 | 2200 ± 1400 | 6300 ± 8300 | primary |
| α-MCA        | 12 ± 9 | 8.6 ± 3.1 | 10 ± 5.8 | primary |
| β-MCA        | 74 ± 51 | 42 ± 25 | 76 ± 75 | primary |
| ω-MCA        | 63 ± 27 | 37 ± 12 | 33 ± 11 | primary |
| HCA          | 1.5 ± 1.3 | 2.4 ± 2.2 | 1.0 ± 0.84 | primary |
| LCA          | 81 ± 30 | 96 ± 36 | 100 ± 48 | secondary |
| UDCA         | 160 ± 84 | 100 ± 37 | 120 ± 92 | secondary |
| DCA          | 2600 ± 1600 | 1200 ± 470 | 2100 ± 2100 | secondary |
| T-CA         | 29000 ± 49000 | 1800 ± 780 | 16000 ± 31000 | conjugated primary |
| T-α-MCA      | 1200 ± 2000 | 330 ± 230 | 1900 ± 3900 | conjugated primary |
| T-β-MCA      | 6600 ± 12000 | 900 ± 590 | 9800 ± 22000 | conjugated primary |
| T-ω-MCA      | 2400 ± 3900 | 500 ± 360 | 2400 ± 4900 | conjugated primary |
| T-HCA        | 32 ± 44 | 13 ± 6.2 | 62 ± 110 | conjugated primary |
| T-DCA        | 1200 ± 1700 | 290 ± 110 | 830 ± 1100 | conjugated secondary |

Data are given as mean ± SD. n=8 animals in each group for feces samples; n=8 animals for plasma CHD group; n=7 animals for plasma WD+vehicle and WD+c[wLwReQeR] groups.
Supplementary Table 7. Observed levels of cytokines/chemokines in liver tissue of WD-fed LDLr-/– mice following 4 weeks of treatment with c[wlwReQeR] or vehicle, as determined by Luminex assay.

| Cytokine/chemokine   | WD + vehicle (pg/g tissue) | WD + c[wlwReQeR] (pg/g tissue) | p value |
|----------------------|---------------------------|---------------------------------|---------|
| CCL2 (MCP-1)         | 1100 ± 350                | 470 ± 150                       | 0.02    |
| CCL2 (MIP-2)         | 1000 ± 290                | 460 ± 110                       | 0.01    |
| CCL3 (MIP-1a)        | 2200 ± 1900               | 200 ± 130                       | 0.09    |
| CCL4 (MIP-1b)        | 17000 ± 9400              | 3000 ± 1800                     | 0.02    |
| CXCL10 (IP-10)       | 2100 ± 590                | 1200 ± 380                      | 0.04    |
| Eotaxin              | 1700 ± 1100               | 1200 ± 390                      | 0.4     |
| G-CSF                | 380 ± 55                  | 380 ± 150                       | 1.0     |
| GM-CSF               | 140 ± 88                  | 86 ± 37                         | 0.3     |
| IFNg                 | 56 ± 25                   | 37 ± 16                         | 0.3     |
| IL-12p40             | 270 ± 100                 | 200 ± 78                        | 0.3     |
| IL-12p70             | 270 ± 42                  | 270 ± 140                       | 1.0     |
| IL-15                | 840 ± 140                 | 960 ± 370                       | 0.5     |
| IL-17                | 67 ± 12                   | 55 ± 16                         | 0.2     |
| IL10                 | 170 ± 44                  | 160 ± 58                        | 0.7     |
| IL13                 | 5.9 ± 4.2                 | 7.3 ± 5.9                       | 0.7     |
| IL1a                 | 2900 ± 540                | 1100 ± 190                      | 0.0004  |
| IL1b                 | 610 ± 130                 | 430 ± 100                       | 0.05    |
| IL2                  | 120 ± 32                  | 80 ± 24                         | 0.1     |
| IL3                  | 34 ± 8                    | 31 ± 15                         | 0.7     |
| IL4                  | 13 ± 2.3                  | 13 ± 8.4                        | 1.0     |
| IL5                  | 40 ± 9.1                  | 39 ± 25                         | 1.0     |
| IL6                  | 410 ± 120                 | 260 ± 55                        | 0.04    |
| IL7                  | 220 ± 52                  | 230 ± 110                       | 0.9     |
| IL9                  | 1100 ± 240                | 670 ± 160                       | 0.01    |
| KC                   | 450 ± 200                 | 190 ± 20                        | 0.04    |
| LIF                  | 8.6 ± 2.8                 | 7.1 ± 4                         | 0.5     |
| LIX                  | 320 ± 370                 | 290 ± 240                       | 0.9     |
| M-CSF                | 260 ± 50                  | 200 ± 70                        | 0.2     |
| MIG                  | 1800 ± 660                | 1200 ± 650                      | 0.2     |
| RANTES               | 170 ± 110                 | 71 ± 61                         | 0.1     |
| TNFα                 | 110 ± 20                  | 77 ± 21                         | 0.03    |
| VEGF                 | 13 ± 4.2                  | 8.2 ± 3.4                       | 0.1     |

Data are given as mean ± SD. n=5 animals for WD group; n=4 animals for peptide-treated WD group. p values were determined by two-tailed Student's t-test.
## Supplementary Table 8. Primers for qPCR experiments.

| Gene name | Forward (5’-3’) | Reverse (5’-3’) |
|-----------|-----------------|-----------------|
| 16S       | CCGCAAGGGAAAGATGAAAGAC | TCGTTTGTTTCGGGGTTTC |
| β-actin   | ATGGAGGGAATACACGCCC | TTCTTTGCAAGCTCTCCTCCTTT |
| Cyp7a1    | AGCAACTAAACAACCTGCCGATCTA | GTCCGGATATTCAAGGATGCA |
| Cyp8b1    | GGCTGGCTTCCTGAGCTTATT | ACTTCCCTGAACAGCTCATCGG |
| Cyp27a1   | GCCTCACCTATGGGATCTTCA | TCAAAGCCTGACGACGATG |
| Akr1d1    | TGCACACCACACAAATATCCCT | CTCTACTGCCACACATCGGCTT |
| Mrp2      | TCTGTGAGTGCAAGAGACAGGT | TCCAGGACCAAGAGATTTTC |
| Mrp3      | CCGAAACTACGCACCAGATG | GATGGGCTGGCTATTGTGCTG |
| Asbt      | TGGTGTAGACGAAGAGGGGAA | GCCTATGGATAGAGGACGA |
| Fgf15     | CAGTCTTCCTCCGAGTAGCG | TGAAGACGATTGAGCCCAAG |
| Tjp1      | CCTGTGAAGCGTACACTGTG | CGCGGAGAGAGACAAGATGT |
| Ocln      | CATAGTCAGATGGGGTGGA | ATTTATGATGAACAGCCCAT |

