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Supplementary Information for

Paneth cell α-Defensins HD-5 and HD-6 display differential degradation in active antimicrobial fragments

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Supplementary Information Text

Material and Methods

Bacterial strains

A. baumannii 4-MRGN, *K. pneumoniae* 4-MRGN, *P. aeruginosa* ATCC27853, *E. faecium* 475747, *B. longum*, *L. fermentum*, *L.* salivarius and S. *salivarius salivarius* were obtained as clinical isolates from the Robert-Bosch-Hospital (Stuttgart, Germany). *Akkermansia muciniphila*, *B. subtilis* 168trpC and *S. aureus* USA300 were received from the Institut für Mikrobiologie und Infektionsmedizin (Tübingen, Germany). *B. adolescentis* Ni3,29c, *B. breve* were provided by Ardeypharm. *B. vulgatus* DSM1447 was obtained from DSMZ and *L. rhamnosus* were provided by InfektoPharm (Heppenheim, Germany).

Peptides

For all experiments oxidized peptides HD-5 and HD-6 (Peptide Institute, Osaka, Japan) were used. All fragments HD-5_{1-9}, HD-5_{1-13}, HD-5_{1-28}, HD-5_{7-32}, HD-5_{10-32}, HD-5_{14-32}, HD-5_{10-27} and HD-5_{26-32} were synthesized by EMC microcollections GmbH (Tübingen, Germany). All peptides were dissolved in 0.01% acetic acid (HAc) in similar concentrations.

Collection of duodenal fluid during gastroscopy

The human duodenal fluid was collected during a routine gastroscopy from three healthy individuals. The duodenum was washed with 0.9% NaCl solution, which was recollected. Patients gave their written and informed consent after they were informed. The sample collection had been previously approved by the Ethical Committee of the University Hospital of Tübingen, Germany.

Screening for fragments of HD-5 and HD-6 using LC/MS

We incubated 2.5 µg of HD-5 or HD-6 in 50 mM NH_4HCO_3 buffer (pH 8.0) (Fluka) with 2 mM *tris* (2-carboxyethyl) phosphine for 15 minutes at 37°C. Afterwards we added human duodenal fluid and incubated it for additional 30 minutes at 37°C. At last, we added formic acid and acetonitrile in a final concentration of 0.5% and 10%, respectively, and analyzed the samples by mass spectrometry. Mass spectrometry was performed as a LC/MS system using an Agilent 1200 series HPLC with an Agilent Advanced Bio Peptide Map (2.1x150 mm, 2.7 µm) column with a flow of 0.4 ml/min at 55°C column temperature and a 6540 UHD Q-TOF LC/MS system (Agilent) for mass analysis. The samples were separated by a gradient of acetonitrile in 0.1% formic acid. The gradient started at 2% acetonitrile for 4 minutes and then increases during 35 minutes to 45%. Mass spectrometric analyses were performed in single MS mode from 100 to 3400 m/z with positive ion polarity and were analyzed by Agilent MassHunter Quantitative Analysis B 06.00 software.

Scanning-Electron-Microscopy

Scanning-Electron-Microscopy was performed as previously described[1]. Briefly, Protein A coated beads (Spherotech Inc.) were incubated with reduced HD-6 (200 µg/ml) in 10 mM sodium phosphate buffer with 1% (w/v) TSB for 1.5 hours at 37°C to allow net formation. We

subsequently incubated the whole sample with duodenal fluid for additional 30 minutes at 37°C. As a control we used 0.01% acetic acid (HAc). Beads were centrifuged and fixed in Karnovsky's reagent. The samples were washed with PBS and additional fixed with 1% OsO_4 in H_2O. Then, they were dehydrated to 100% ethanol and critical point dried from CO_2 and analyzed by scanning electron microscopy at the Max Planck Institute for Developmental Biology (Tübingen, Germany).

Transmission Electron Microscopy

Transmission electron microscopy experiments were performed as previously described[2]. 6 x 10^8 cfu *E. coli* MC1000 was incubated with 200 µg/ml of each peptide for 2 hours. Bacteria were fixed in Karnovsky's fixative, embedded in agarose, coagulated, cut in small blocks and fixed again in Karnovsky's solution. After post-fixation and embedding in glycid ether blocks were cut using an ultra-microtome. Sections (30 nm) were mounted on copper grids and analyzed using a Zeiss LIBRA 120 transmissions electron microscope.

Radial diffusion assay

Antimicrobial activity of all peptides were tested with a modified radial diffusion assay from *Lehrer et al.*[3]. Shortly described, log-phase bacteria grew (anaerobic bacteria with AnaeroGen, Oxoid in anaerobic jars) in liquid tryptic soy broth (TSB) (Becton Dickinson). After several wash steps in 10 mM sodium phosphate buffer pH 7.4, 4 x 10^6 cfu/ml was used per assay. To measure the antibacterial effect of the identified peptide fragments, the bacteria was incubated in 10 mM sodium phosphate (pH 7.4) containing 0.3 mg/ml TSB powder and 1 % (w/v) low EEO-agarose (Applichem). Peptide fragments were then pipetted into punched wells and allowed to diffuse for 3 hours at 37°C. After that a nutrient rich gel with 6 % TSB (w/v) and 1 % agarose in 10 mM sodium phosphate buffer was poured on top of the first gel. After 24 hours the inhibition zones were measured. We used 0.01 % acetic acid as a negative control, which did not show inhibition zones greater than the diameter of the punched well. All experiments were carried out at least three times.

Turbidity broth assay

The tested bacteria were incubated overnight in 1x TSB broth, centrifuged and washed with 10 mM sodium phosphate buffer containing 1% (w/v) TSB broth. 5 x 10^5 cfu/ml bacteria were mixed with different peptide concentrations in 10 mM sodium phosphate buffer with 1% (w/v) TSB (final volume 100 µl) and incubated for 2 hours at 37 °C. Afterwards we added 100 µl 2x TSB broth and we measured the optical density at 600 nm (Spark 10M, Tecan, Austria). Bacterial growth was monitored for 12 hours, growing at 37 °C with shaking during the measurements each 30 minutes, except for *Akkermansia muciniphila* which were incubated at 37 °C in an anaerobic jar and growth was measured after 72 hours.

***In vivo* microbiota analysis**

To assess proof of concept of a functional impact on microbiota composition, we administered HD-5_{1-9} to 9 weeks old healthy chow fed male mice housed in groups of 3 per cage. Mice were acclimatized for 3 weeks prior to the study and stratified into experimental groups based on body weight, ensuring equal weight distribution between groups. In more detail, we treated wild-type

C57BL/6J mice by oral gavage for 7 days with 7.19µg/mouse HD-5_{1-9} administered in 100µL PBS solution. Control mice were treated with equal volume of PBS. We initially performed a 7-day experiment with 6 mice per group. Based on these results, we designed a new study, also including 6 mice per group, to study the temporal impact of gut microbial modulations. In this study, we included a 7 days wash period, after 1 week of oral gavage. Fresh feces samples were collected from individual mice at day 0, 7 and 14 (treated and control group n = 6 in each) at 9AM at the same time body weight was measured. At day 14, mice were euthanized and the small intestine content was collected. All animal protocols were conducted according to guidelines set out by the Laval University Animal Care and Handling Committee. C57BL/6J male mice (Jackson Laboratories, Bar Harbor, ME) were housed in a pathogen-free, temperature-controlled environment under a 12:12 hour light-dark cycle and fed ad libitum standard rodent chow diet (Harlan Teklad T-2018) for the 5 weeks of accommodation in our animal facility (3 weeks of acclimatization and 2 weeks of experimental protocol).

Bacterial DNA was extracted from snap-frozen feces and content of small intestine at necropsy by the NuceloSpin 96 soil kit (Macherey-Nagel) following the manufacturer's instructions. BGI, Europe performed the subsequent library preparation and DNA sequencing using in-house standard operating procedures. In brief, 30 ng of bacterial DNA per sample was PCR amplified using the primers: 515F:GTGCCAGCMGCCGCGGTAA, 806R:GGACTACHVGGGTWTCTAAT with Illumina adapters targeting the V4 16S rDNA region. PCR products were then purified with AmpureXP beads (AGENCOURT) to remove unspecific products. The average molecule length was determined by Agilent 2100 bioanalyzer (Agilent DNA 1000 Reagents). DNA quantification was evaluated by real-time quantitative PCR (EvaGreen^{TM}) before pair end sequencing on a HiSeq2500 system.

Processing and quality control of reads was performed using the R package DADA2, version 1.4.0[4] and forward and reverse primers were trimmed off from reads. Next, all reads containing remaining uncalled bases or more than two expected errors were removed.

Afterward, the parameters of the DADA2 error model were learned from a random subset of 1 million reads. This error model was then used to denoise all sequences; i.e., to infer the ASVs. Denoised reads (ASVs) were then merged and read pairs with one or more conflicting bases between the forward and reverse read were removed. ASVs shorter than 251 and longer than 254 bases were discarded. Chimeric sequences were then detected and removed using the function "removeBimeraDenovo." Finally, reads (ASVs) were classified from the kingdom to the genus level using the Silva reference *16S rRNA* gene database, version 132 resulting in the construction of an ASV table with read counts of all ASVs in all samples.

Statistical analysis

Apart from microbiota analyses, all data were analyzed with GraphPad Prism 7. Values of $p < 0.05$ were considered as statistically significant. All results are depicted as mean and their ± standard deviation, with their standard error of the mean or as 80 % confidence interval, as indicated in the figure legend. Bioinformatical analysis was carried out using R Studio (R version 3.4.2 and R Studio version 1.0.136) and packages phyloseq 1.22.3[5], metagenomeSeq 1.20.0[6], vegan 2.4-4[7], lme4 1.1-15, ggplot2 2.2.1[8] and GUniFrac 1.1[9]. For the linear mixed model (lme), we included a cut-off of 0.1% in relative abundance and only considered microbes that were present in ≥ two samples. Longitudinal sampling of fresh fecal samples were evaluated by paired

analyzes; for single endpoint samples from the small intestine we used cage-adjusted p-values to account for potential cage effects of group housed mice. The distance in microbiota composition from baseline was made using the GUniFrac package calculating Weighted and Unweighted UniFrac distances from each sample to its baseline composition.

Software

For our *in silico* digest analysis we used the ExPASy PeptideMass tool from the SIB Bioinformatics Resource Portal. We used trypsin, chymotrypsin or both in combination for our *in silico* digest of HD-5 or HD-6 with a maximum of 5 missed cleavages and fragments bigger than 500 Da. All fragments were listed as their monoisotopic mass as $[M+H]^+$. https://web.expasy.org/peptide_mass/

Supporting References

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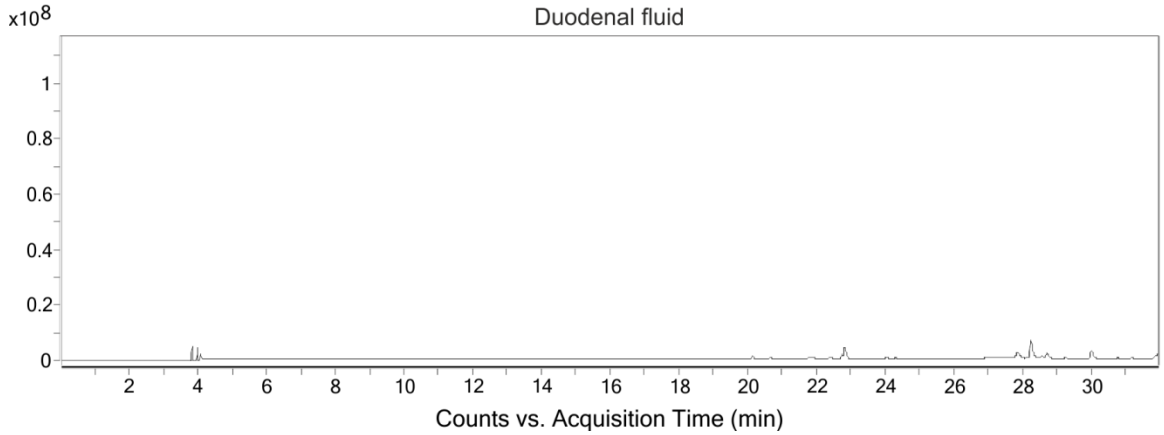

Fig. S1. **Chromatogram of the obtained duodenal fluid used as digestive agent for HD-5/-6**
Here we show the chromatogram of the obtained duodenal fluid, we used to digest the Paneth Defensins. We were not able to detect any HD-5 or HD-6 related fragments, therefore all fragments shown in Figure 1 and 2 are originated of the digestion from HD-5 or HD-6 as described in the figure legends.

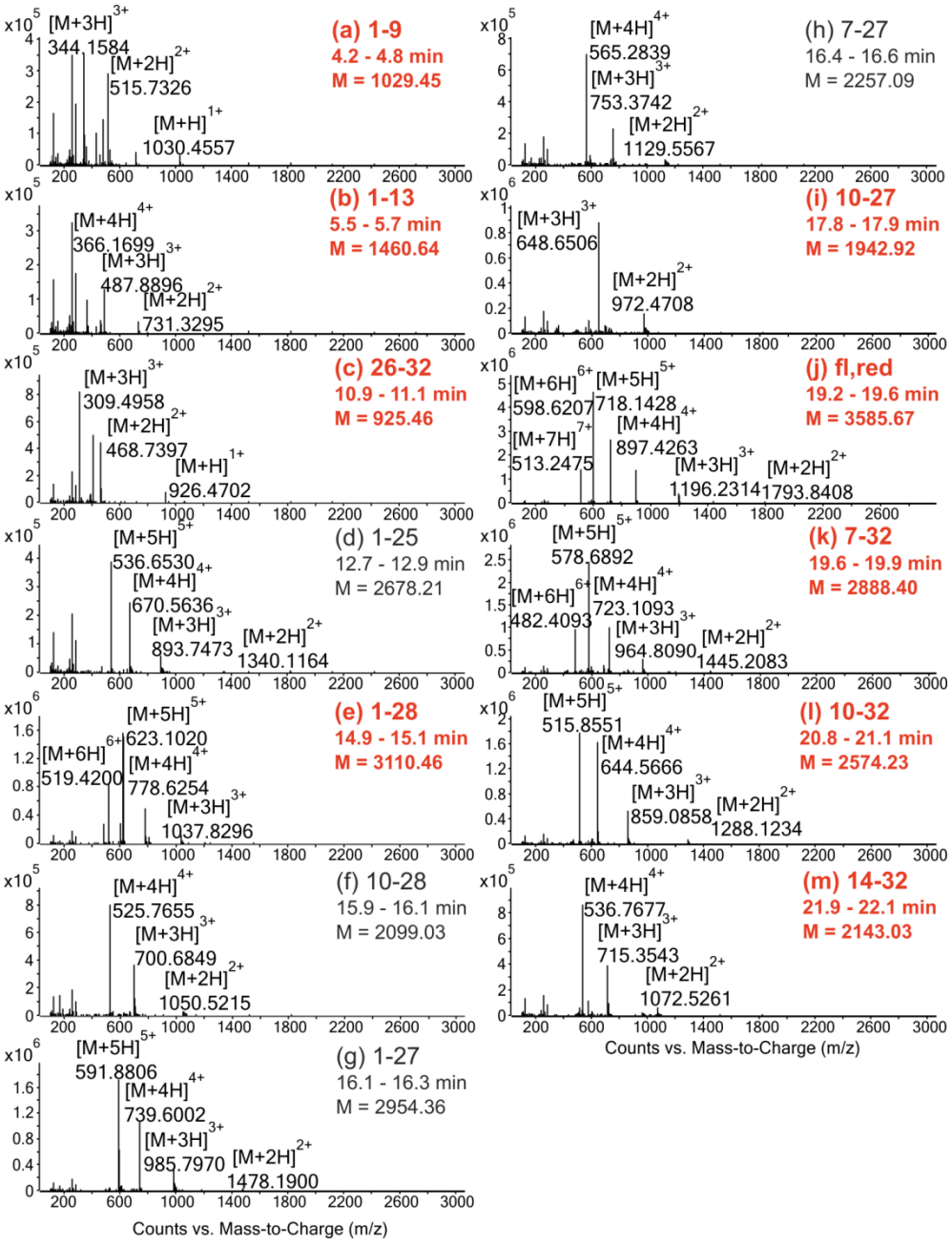

Fig. S2. **Incubation of HD-5 and duodenal fluid led to many different fragments**

Here we show the mass-to-charge (m/z) graphs of all identified fragments from Figure 2 with the detected ions and their neutral masses. All peptides marked in red were chose for synthesis and deeper investigation of their abilities.

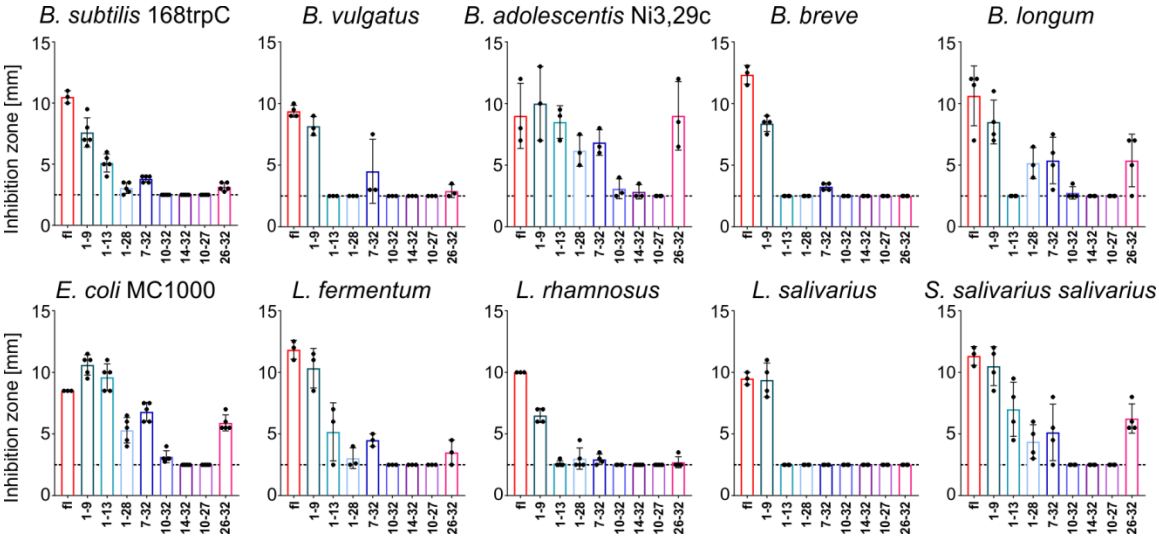

Fig. S3. **HD-5 fragments are antimicrobial active against commensal bacteria**
Here we show the dataset the heatmap of Figure 3 is based on.

A

pathogenic bacteria	HD-5								
	fl	1-9	1-13	1-28	7-32	10-32	14-32	10-27	26-32
A. baumannii 4-MRGN									
K. pneumoniae 3-MRGN									
P. aeruginosa ATCC 27853									
E. faecium 475747									
S. aureus USA300									

high activity | low activity | no activity

B

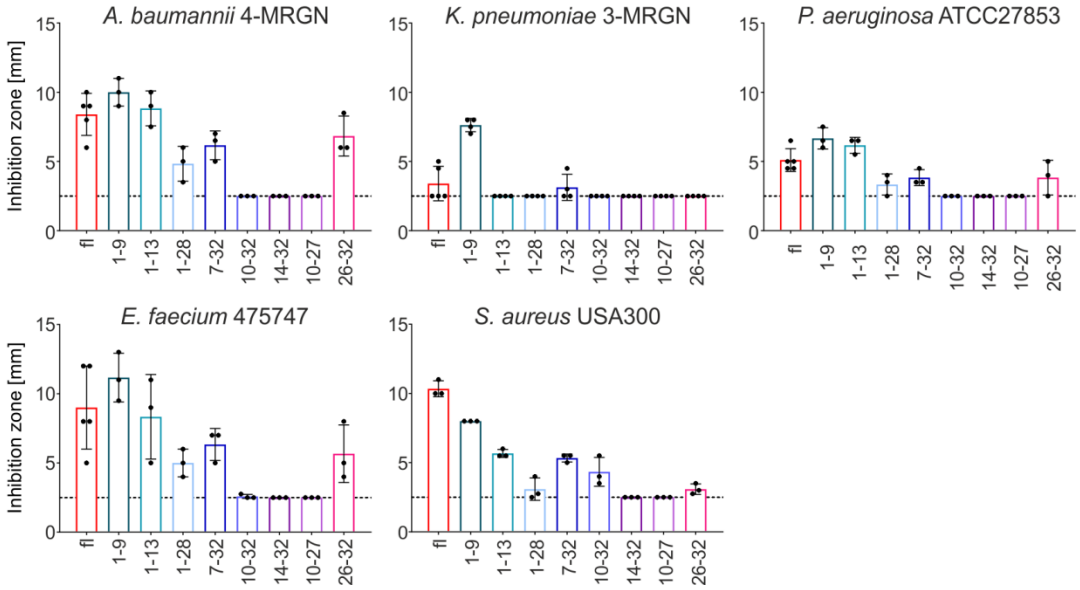

Fig. S4. **HD-5 fragments are antimicrobial active against pathogenic bacteria**
(A) Here we tested the antimicrobial activity of HD-5 fragments (2 µg of HD-5_{fl} and 4 µg of each fragment) against pathogenic bacteria. We used a heat map system with high activity (inhibition zone in the RDA > 5 mm), low activity (2.5 to 5 mm) and no activity (2.5 mm). **(B)** Shows the data of **(A)** with mean and standard deviation from at least three independent experiments.

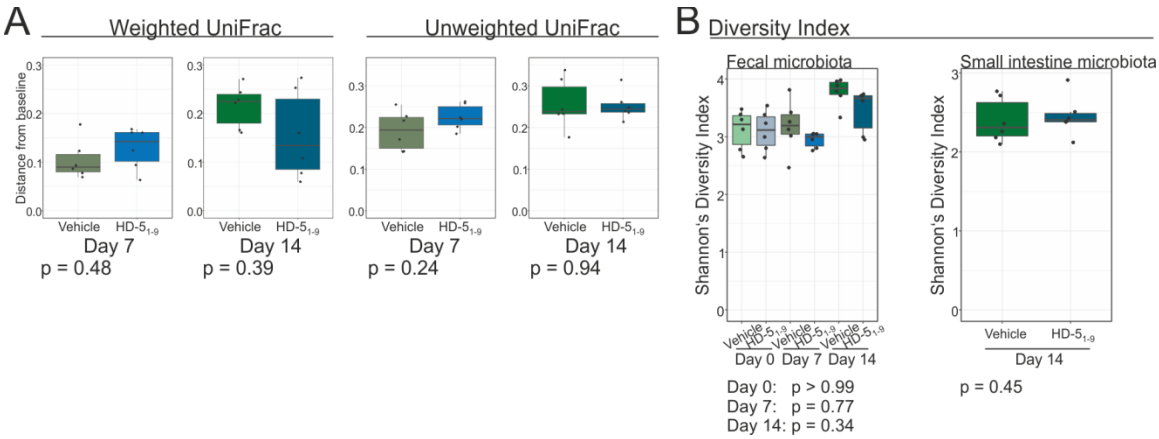

Fig. S5. **HD-5$_{1-9}$ treatment did not affect the microbiota diversity but has an influence to certain bacteria strains**

Here we show supporting information to Figure 4. (A) Weighted/Unweighted UniFrac Distance from the baseline microbiota was calculated using the distance from Day 0 to Day 7 and Day 0 to Day 14 from the individual mice. The distances were not significantly different between the groups at any timepoint regardless of the used distance method (Adonis PERMANOVA $p > 0.05$). **(B)** Fecal and small intestine microbiota was calculated by Shannon's Diversity index. Data from fecal samples followed Gaussian Distribution and were analyzed with 2-way ANOVA RM, adjusted by Bonferroni's test for multiple comparisons. Data from the small intestine did not follow Gaussian Distribution and were accordingly evaluated by Wilcoxon rank sum test.

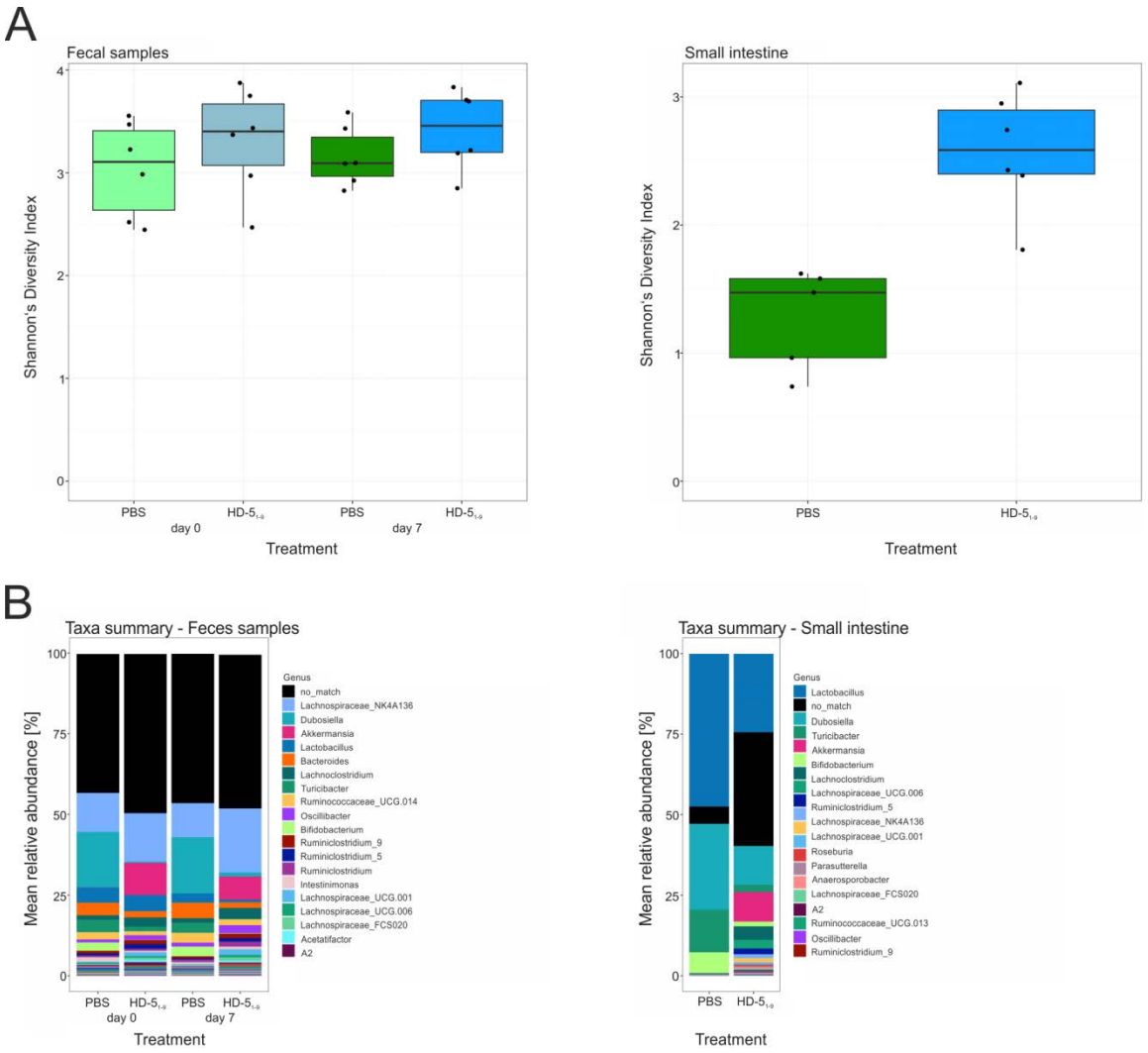

Fig. S6. **HD-5_{1-9} treatment increases the microbiota diversity in the small intestine but not in feces samples**
We treated mice orally for 7 days with HD-5_{1-9} (6 mice per group) and collect feces and small intestine samples to investigate the microbiota composition. **(A)** Fecal and small intestine microbiota diversity was calculated by Shannon's Diversity index. We did not observe a significant difference between the HD-5_{1-9} and PBS treatment after day 7 in feces samples ($p = 0.18$, Wilcoxon test), but in the small intestine ($p = 0.004$; Wilcoxon test). Our results showed that the small intestine microbiota diversity increases after seven days of HD-5_{1-9} treatment. **(B)** HD-5_{1-9} treatment affects the abundance of different bacterial genera. Using a linear mixed-effect model, we identified a significant effect of the treatment on *Akkermansia sp.* in the feces (Linear mixed model, $p < 0.001$) and small intestine samples (Linear mixed model, $p = 0.002$).

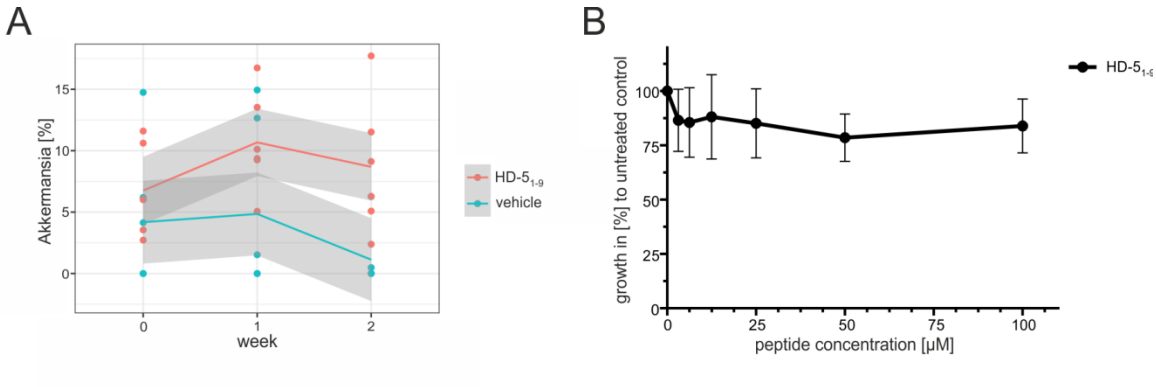

Fig. S7. **Abundance of *Akkermansia* in feces is increased in HD-5$_{1-9}$ treated mice, while it is not susceptible to HD-5$_{1-9}$ treatment in turbidity assay.**
(A) The amount of *Akkermansia sp.* in feces samples (day 0, 7, 14) collected from mice treated for 7 days with HD-5$_{1-9}$ or PBS is increased in HD-5$_{1-9}$ treated animals compared to PBS treated ones (linear mixed-effect model; $p = 0.024$). Here we show the mean with the 80% confidence interval from n=6 per group. In **(B)** we tested if *Akkermansia muciniphila* is susceptible to HD-5$_{1-9}$ treatment. Here we show the growth rate compared to an untreated control in % after 72 hours of incubation at 37 °C in an anaerobic jar. We could not detect a MIC from HD-5$_{1-9}$ against of *Akkermansia* in this assay. The graph shows the mean with standard deviation of n=3.

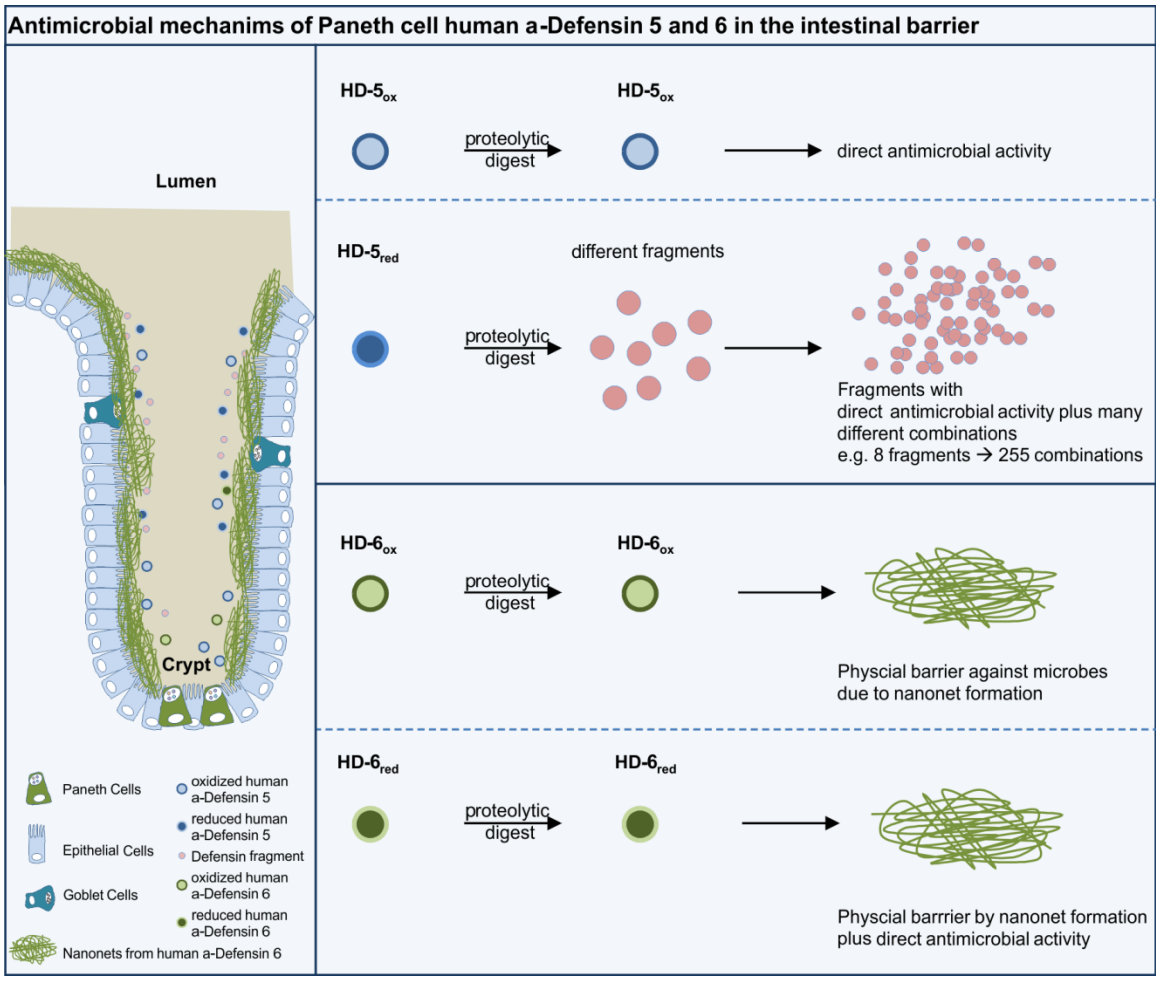

Fig. S8. **Summary of Paneth cell defensins mediated antimicrobial defense mechanisms.**
Here we show the different kinds of antimicrobial defense mechanism of Paneth cell defensins. While HD-5_{ox} has a direct antimicrobial activity, HD-5_{red} is digested by proteases leading to different, still antimicrobial active, fragments. In contrast to HD-5, neither HD-6_{ox} nor HD-6_{red} is susceptible to proteolytic digestion. While HD-6_{ox} and HD-6_{red} are able to form nanonets to entrap bacteria, only HD-6_{red} showed an additional direct antimicrobial activity. All of these mechanisms are working together to protect the epithelial surface as schematically shown in the left part.

Table S1. ***In silico*** **digest of HD-5 and HD-6 with intestinal proteases**

HD-5			HD-6		
position	**missed cleavages**	**sequence**	**position**	**missed cleavages**	**sequence**
1-32	**5**	**ATCYCRTGRCATRESLSGVCEISGRLYRLCCR**	**1-32**	**3**	**AFTCHCRRSCYSTEYSYGTCTVMGINHRFCCL**
1-29	4	ATCYCRTGRCATRESLSGVCEISGRLYRL	3-32	5	TCHCRRSCYSTEYSYGTCTVMGINHRFCCL
5-32	4	CRTGRCATRESLSGVCEISGRLYRLCCR	1-29	5	AFTCHCRRSCYSTEYSYGTCTVMGINHRF
1-28	**4**	**ATCYCRTGRCATRESLSGVCEISGRLYR**	1-28	2	AFTCHCRRSCYSTEYSYGTCTVMGINHR
1-27	**3**	**ATCYCRTGRCATRESLSGVCEISGRLY**	3-29	4	TCHCRRSCYSTEYSYGTCTVMGINHRF
7-32	**4**	**TGRCATRESLSGVCEISGRLYRLCCR**	3-28	5	TCHCRRSCYSTEYSYGTCTVMGINHR
1-26	2	ATCYCRTGRCATRESLSGVCEISGRL	8-32	2	RSCYSTEYSYGTCTVMGINHRFCCL
5-29	3	CRTGRCATRESLSGVCEISGRLYRL	9-32	1	SCYSTEYSYGTCTVMGINHRFCCL
1-25	**3**	**ATCYCRTGRCATRESLSGVCEISGR**	1-23	4	AFTCHCRRSCYSTEYSYGTCTVM
5-28	5	CRTGRCATRESLSGVCEISGRLYR	8-29	5	RSCYSTEYSYGTCTVMGINHRF
10-32	**3**	**CATRESLSGVCEISGRLYRLCCR**	3-23	3	TCHCRRSCYSTEYSYGTCTVM
5-27	2	CRTGRCATRESLSGVCEISGRLY	8-28	1	RSCYSTEYSYGTCTVMGINHR
7-28	3	TGRCATRESLSGVCEISGRLYR	9-29	4	SCYSTEYSYGTCTVMGINHRF
5-26	1	CRTGRCATRESLSGVCEISGRL	12-32	4	STEYSYGTCTVMGINHRFCCL
7-27	**3**	**TGRCATRESLSGVCEISGRLY**	9-28	0	SCYSTEYSYGTCTVMGINHR
5-25	3	CRTGRCATRESLSGVCEISGR	1-17	3	AFTCHCRRSCYSTEYSY
14-32	**2**	**ESLSGVCEISGRLYRLCCR**	12-29	3	STEYSYGTCTVMGINHRF
10-28	**2**	**CATRESLSGVCEISGRLYR**	12-28	2	STEYSYGTCTVMGINHR
7-25	2	TGRCATRESLSGVCEISGR	16-32	3	SYGTCTVMGINHRFCCL
10-27	2	CATRESLSGVCEISGRLY	3-17	2	TCHCRRSCYSTEYSY
17-32	3	SGVCEISGRLYRLCCR	1-15	2	AFTCHCRRSCYSTEY
1-16	1	ATCYCRTGRCATRESL	18-32	2	GTCTVMGINHRFCCL
14-28	1	ESLSGVCEISGRLYR	3-15	1	TCHCRRSCYSTEY
10-25	1	CATRESLSGVCEISGR	16-29	2	SYGTCTVMGINHRF
14-27	1	ESLSGVCEISGRLY	16-28	1	SYGTCTVMGINHR
1-13	**2**	**ATCYCRTGRCATR**	1-11	1	AFTCHCRRSCY
17-29	2	SGVCEISGRLYRL	12-23	2	STEYSYGTCTVM
5-16	0	CRTGRCATRESL	18-29	1	GTCTVMGINHRF
14-25	0	ESLSGVCEISGR	8-17	3	RSCYSTEYSY
17-27	1	SGVCEISGRLY	18-28	0	GTCTVMGINHR
1-9	**1**	**ATCYCRTGR**	3-11	2	TCHCRRSCY
5-13	2	CRTGRCATR	9-17	2	SCYSTEYSY
17-26	0	SGVCEISGRL	24-32	1	GINHRFCCL
26-32	**1**	**LYRLCCR**	8-15	2	RSCYSTEY
27-32	2	YRLCCR	1-8	2	AFTCHCRR
7-13	1	TGRCATR	16-23	1	SYGTCTVM
1-6	0	ATCYCR	9-15	1	SCYSTEY
28-32	1	RLCCR	1-7	0	AFTCHCR
5-9	1	CRTGR	3-8	1	TCHCRR
			12-17	1	STEYSY
			24-29	0	GINHRF
			3-7	0	TCHCR
			18-23	0	GTCTVM
			8-11	1	RSCY

In silico (without color) and *ex vivo* reality after duodenal mucus incubation (underlined in blue or red) digest of Paneth cell HD-5 and HD-6 with trypsin or chymotrypsin or both in combination, maximum of 5 missed cleavages and fragments bigger than 500 Da identified as their monoisotopic mass as $[M+H]^+$ using the ExPASy PeptideMass module. Fragments which can be identified with mass spectrometry after incubation of the human peptides with human duodenal mucus are bold and underlined in blue. Underlined in red are the two full-length peptides, which could be identified too.

Table S2. **HD-5 fragments are able to inhibit the growth of pathogenic bateria**

		Bacteria									
		A. baumannii 4-MRGN		*K. pneumoniae* 3-MRGN		*P. aeruginosa* ATCC 27853		*E. faecium* 475747		*S. aureus* USA300	
Peptides		MIC in µM	MIC in µg/ml	MIC in µM	MIC in µg/ml	MIC in µM	MIC in µg/ml	MIC in µM	MIC in µg/ml	MIC in µM	MIC in µg/ml
HD-5	fl	6,25	22.4	---	---	25	89.7	3,125	11.2	3,125	11.2
	1-9	25	25.75	---	---	50	51.5	12,5	25.75	50	51.5
	1-13	> 100	> 146	---	---	> 100	> 146	---	---	---	---
	1-28	> 100	> 311	---	---	> 100	> 311	100	311	---	---
	7-32	25	72.25	---	---	50	144.5	25	72.2	---	---
	10-32	> 100	> 257	---	---	> 100	> 257	100	257	---	---
	14-32	---	---	---	---	> 100	> 214.4	---	---	---	---
	10-27	---	---	---	---	---	---	---	---	---	---
	26-32	---	---	---	---	---	---	---	---	---	---

Each experiment was carried out at least three times. We determine the MIC in µM and µg/ml as the concentration without any bacterial growth in every experiment after 12 hours of incubation. If no influence or MIC was detectable we marked it with "---".