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Exploring the therapeutic potential of endolysin CD27L_EAD against *Clostridioides difficile* infection

Youngjin Cho[a], Kyungah Park[a], Jeongseok Park[a], Jieun An[a], Heejoon Myung[b,c], Hyunjin Yoon[a,d,*]

[a] *Department of Molecular Science and Technology, Ajou University, Suwon, South Korea*
[b] *LyseNTech Co., Ltd., Seongnam, South Korea*
[c] *Department of Bioscience and Biotechnology, Hankuk University of Foreign Studies, Yongin, South Korea*
[d] *Department of Applied Chemistry and Biological Engineering, Ajou University, Suwon, South Korea*

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ABSTRACT

Objectives: *Clostridioides difficile* has emerged as a major cause of life-threatening diarrheal disease. Conventional antibiotics used in current standards of care exacerbate the emergence of antibiotic-resistant strains and pose a risk of recurrent *C. difficile* infection (CDI). Thus, there is an urgent need for alternative therapeutics that selectively eliminate *C. difficile* without disturbing the commensal microbiota. This study aimed to explore the potential of endolysins as an alternative therapeutic agent to antibiotics. Endolysin is a bacteriophage-derived peptidoglycan hydrolase that aids in the release of phage progeny during the final stage of infection.

Methods: In order to exploit endolysin as a therapeutic agent against CDI, the bactericidal activity of 23 putative endolysins was compared and ΦCD27 endolysin CD27L was selected and modified to CD27L_EAD by cleaving the cell-wall binding domain of CD27L.

Results: CD27L_EAD exhibited greater bacteriolytic activity than CD27L and its activity was stable over a wide range of salt concentrations and pH conditions. CD27L_EAD was added to a co-culture of human gut microbiota with *C. difficile* and the bacterial community structure was analyzed. CD27L_EAD did not impair the richness and diversity of the bacterial population but remarkably attenuated the abundance of *C. difficile*. Furthermore, the co-administration of vancomycin exerted synergistic bactericidal activity against *C. difficile*. β-diversity analysis revealed that CD27L_EAD did not significantly disturb the composition of the microbial community, whereas the abundance of some species belonging to the family Lachnospiraceae decreased after CD27L_EAD treatment.

Conclusions: This study provides insights into endolysin as a prospective therapeutic agent for the treatment of CDI without damaging the normal gut microbiota.

1. Introduction

Clostridioides difficile is a spore-forming gram-positive anaerobe implicated in antibiotic-associated diarrhea, particularly nosocomial diarrhea [1]. Although *C. difficile* is commonly found in the gastrointestinal tract, the indigenous microbiota hinders *C. difficile* from colonizing favorable niches, a phenomenon termed colonization resistance [2], and protects healthy individuals from *C. difficile* infection (CDI). The incidence of CDI has increased globally over the last two decades [3] and a recent meta-analysis estimates the incidence of hospital-onset CDI was 8.3 cases per 10 000 patient-days [4]. Its prevention in healthcare settings has become an important public health priority worldwide. The established international standards for CDI treatment rely on conventional antibiotics such as vancomycin, fidaxomicin, and metronidazole [1,5]. However, antibiotic therapy using broad-spectrum antibiotics poses many challenging obstacles, including gut microbiota dysbiosis, recurrent CDI, and the emergence of antibiotic-resistant and hypervirulent *C. difficile* strains [6,7]. Due to the concerning outcome from long-term use of broad-spectrum antibiotics, alternative therapeutic approaches have been explored, including phage-based therapy [8,9], fecal microbiota transplantation (FMT) [10], and fecal virome transplantation (FVT) [11].

* Corresponding author: Hyunjin Yoon, Department of Applied Chemistry and Biological Engineering, Ajou University, Suwon 16499, South Korea. Tel.: +82 31 219 2450; fax: +82 31 219 1610
E-mail address: yoonh@ajou.ac.kr (H. Yoon).

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Phages have been extensively studied as therapeutic agents to treat bacterial infections since the project by Felix d'Herelle [12]. However, all *C. difficile*-infecting phages identified to date undergo a lysogenic cycle [13]. It is likely advantageous for phages to integrate into the host genome, given that *C. difficile* prefers the spore form over vegetative cells in hostile environments. Due to the high incidence of prophage-related genetic elements in the host, further viral infections and substantial bacteriolysis are restrictive in *C. difficile*. Instead, phage-derived enzymes, such as endolysins, have been utilized to overcome the drawbacks of phage therapy. Endolysin is produced at the end of the viral replication cycle and lyses the bacterial peptidoglycan layer to release progeny phages [14]. Natural and engineered endolysins devised as enzybiotics have multifaceted advantages over phages and antibiotics, including rapid bacteriolysis, no risk of developing resistance, eradication of multidrug-resistant bacteria, synergistic killing with different antibacterial agents, and biofilm deconstruction [15,16]. The most important feature of endolysins is their narrow spectrum targeting of specific bacterial species, thereby conceptually not disturbing the normal microbiome.

Phages targeting gram-positive bacteria have evolved to use modular endolysins, in which catalytic activity (enzymatic active domain; EAD) and substrate recognition (cell-wall binding domain; CBD) are separated into two distinct types of functional domains [17]. In general, EAD enables the cleavage of Particular bonds within the peptidoglycan layer, while CBD binds specifically to the receptors of bacterial cell walls and anchors them off the lysed cell walls, thereby likely preventing subsequent lysis of neighboring intact bacteria [18]. Therefore, CBD removal occasionally increases the lytic activity of endolysins against gram-positive bacteria [19,20]. Owing to their modular structure, endolysins targeting gram-positive bacteria are worth extensive engineering to improve their lytic activity and host specificity. The present study screened for a promising endolysin to treat CDI and investigated its potential effects on altering the structural composition of normal gut microbiota.

2. Materials and methods

2.1. Endolysin cloning and screening

C. difficile phage endolysin genes (Supplementary Table 1) and their DNAs containing endolysin EADs were synthesized (BIONICS, Seoul, Korea) and amplified by polymerase chain reaction (PCR), respectively, and cloned into pET21a(+) (Novagen, San Diego, CA, USA) at the NdeI and HindIII sites. A His6-tag was fused to the C-terminus of each endolysin for purification. The primers used for recombinant endolysin construction are listed in Supplementary Table 2.

In order to select endolysins, the lysis zone-based rapid screening method [21] was used with minor modifications. Briefly, *E. coli* BL21(DE3) strains harboring each pET21a(+) derivative and pBAD33_SPN1S lysRz [21] were cultivated in Luria-Bertani (LB) broth containing 0.25 mM isopropyl β-D-1-thiogalactopyranoside (IPTG) at 37°C to produce endolysins. Plasmid pET15b_LysB4 [22] was used as a positive control. After 8 h, 0.2% arabinose was added, and the cultures were dotted onto LB agar plates overlaid with autoclaved *C. difficile* cells. The plates were incubated at 37°C to develop clear lysis zones.

2.2. Endolysin purification and bacterial viability and turbidity reduction assays

Escherichia coli BL21(DE3) star or *E. coli* ArcticExpress (DE3) was used for endolysin purification. Bacterial cells were cultivated in LB broth at 25°C and 0.5 mM IPTG was added at the logarithmic growth phase to induce endolysin expression for 4 h. Cells were then harvested and incubated in lysis buffer (20 mM Tris-HCl, pH 7.5, 300 mM NaCl, and 20 mM imidazole) containing 1 mg/mL lysozyme for 30 min. Bacterial cells were disrupted using sonication and centrifuged at 10,000 × *g* for 20 min at 4°C. After filtering the supernatant, the endolysins were purified using Ni-NTA His-tag affinity chromatography and dialyzed in storage buffer (20 mM Tris-HCl, pH 7.5, and 150 mM NaCl).

The bacterial strains used in this study and their growth conditions are listed in Supplementary Table 3. Anaerobic cultivation was performed using an anaerobic workstation (DG250; Don Whitley Scientific, Bingley, UK) filled with 80% N_2, 10% CO_2, and 10% H_2 at 37°C. Aerobic cultivation was conducted with a shaking incubator (IST-4075R; Jeio Tech, Daejeon, Korea) at 37°C. Bacterial cells at the logarithmic phase were resuspended in 20 mM Tris-HCl (pH 7.5) buffer and treated with endolysins at 37°C for 1 h. Turbidity reduction was assessed by measuring the optical density at 600 nm (OD_{600}) with 5 min intervals using a Synergy HTX microplate reader (BioTek, Paramus, NJ, USA). For the viability assay, the cell suspension was diluted using phosphate-buffered saline (PBS) and plated on LB agar plates to count the live cells.

2.3. Fractional inhibitory concentration (FIC) index and quantitative PCR (qPCR)

The FIC index was calculated using minimal inhibitory concentration (MIC) as follows [23]: FICI = (MIC of CD27L_EAD in combination/MIC of CD27L_EAD alone) + (MIC of vancomycin in combination/MIC of vancomycin alone). *C. difficile* ATCC 9689 cells (approximately 10^7 CFU/mL) at the logarithmic phase were mixed with different concentrations of endolsyin (0–640 μM) and vancomycin (0–32 μg/mL) in brain heart infusion (BHI) broth and incubated at 37°C for 24 h. A combination with FICI ≤ 0.5 was considered synergistic [23].

Genomic DNA was extracted using GenElute Bacterial Genomic DNA kits (Sigma, St. Louis, MO, USA) and an aliquot (one-eighth of the total) was mixed with primers (200 nM each) and 2× Thunderbird SYBR qPCR Mix (TOYOBO, Osaka, Japan). qPCR was conducted using StepOne Real-Time PCR System (Applied Biosystems, Foster City, CA, USA): initial denaturation cycle at 95°C for 20 s, followed by 40 cycles at 95°C for 3 s and 60°C for 30 s. Used primers include the toxin A primers specific to *C. difficile* [24] and 341F/R806 primers targeting the V3-V4 region of bacterial 16S rDNA [25].

2.4. Metagenomics

Fecal microbiota (10^7 CFU/mL) from a 32-year-old healthy female was mixed with *C. difficile* ATCC 9689 (10^5 CFU/mL) in BHI broth at a 100:1 ratio and cultivated anaerobically. All the studies involving human subjects were approved by the Ajou University Institutional Review Board (IRB no. 202108-HM-EX-001). Metagenomic DNA was extracted using GenElute Bacterial Genomic DNA kits, and 16S rRNA gene sequencing was performed by CJ Bioscience, Inc. (Seoul, Korea) as detailed in the previous study [26]. Taxonomic alignment was performed using VSEARCH [27] and the EzBioCloud 16S rRNA database [28]. Diversity analysis, including the Shannon α-diversity index [29] and β-diversity distances by Bray–Curtis algorithms [30] was computed using in-house programs from CJ Bioscience, Inc.

2.5. Cytotoxicity assay

Caco-2 cells (HTB-37, ATCC, Manassas, VA, USA) were seeded at 5 × 10^4 cells/well in 96-well plates and incubated with different concentrations of CD27L_EAD from 0 to 320 μM for 24 h. Triton X-100 (1%) and vancomycin (1 μg/mL) were used as positive and

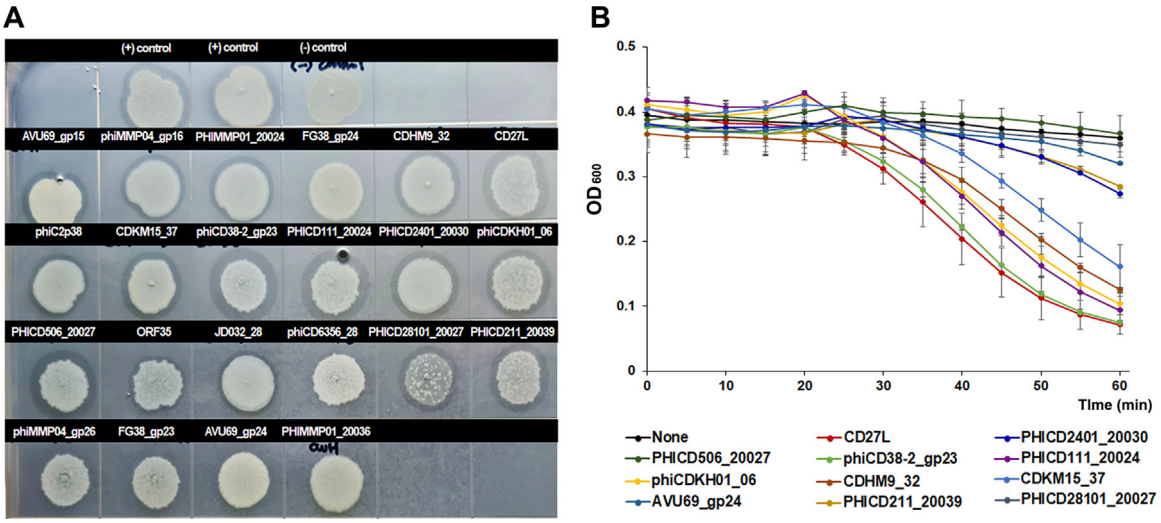

Figure 1. Screening for endolysins against *Clostridioides difficile*. (a) *Escherichia coli* harboring pBAD33_SPN1S lysRz was transformed with pET21a(+) derivatives containing each endolysin gene. Endolysin and SPN1S lysRz were induced by β-D-1-thiogalactopyranoside (IPTG) and arabinose, respectively, and lytic activity was assessed by growing *E. coli* strains on an agar plate overlaid with autoclaved *C. difficile* ATCC 9689 cells. Controls were *E. coli* strains transformed with pET15b_LysB4 and pBAD33_SPN1S lysRz. IPTG was added in both controls, but arabinose was added only in positive controls. (b) *C. difficile* ATCC 9689 cells at logarithmic growth phase were treated with each endolsyin at 0.16 μM in 20 mM Tris-HCl (pH 7.5) buffer and optical density at 600 nm was measured for 1 h. "None" indicates no endolysin was added.

negative controls, respectively. The lactose dehydrogenase (LDH) release assay was conducted to evaluate cell cytotoxicity, using CytoTox 96 Non-Radioactive Cytotoxicity Assay kit (G1780, Promega, Madison, WI, USA), according to the manufacturer's instructions.

2.6. Statistical analysis

Statistical analyses were performed using Prism software (GraphPad Software Inc., San Diego, CA, USA), and statistical differences were analyzed using Student's t-test and one- or two-way ANOVA with Tukey's post-hoc test. Statistical significance was defined as $P < 0.05$.

3. Results

3.1. Selection of six endolysins with high lytic activity against C. difficile

Twenty-three *C. difficile* phage genes predicted to encode peptidoglycan hydrolases were screened for lytic activity on agar plates overlaid with autoclaved *C. difficile* cells (Fig. 1A). Eleven endolysins with clear lysis zones were purified and their bacteriolytic activities were compared using turbidity reduction assays (Fig. 1B). Endolysins with high lytic activity against *C. difficile* were selected as follows: CD27L, phiCD38-2_gp23, PHICD111_20024, phiCDKH01_06, CDHM9_32, and CDKM15_37. Comparing the amino acid sequences divided the six endolysins into two groups with high homology, but all six endolysins were predicted to possess one EAD at their N-terminus and one CBD at their C-terminus (Supplementary Fig. 1). Six endolysins were truncated at their N-terminal EADs, and the lytic activity was compared between the truncated and full-length forms (Fig. 2). CBD removal increase the bacteriolytic activity of each endolysin. Especially, CD27L_EAD exhibited the highest lytic activity.

3.2. Characterization of six recombinant endolysins

The enzymatic activities of six recombinant endolysins lacking cognate CBDs were compared under various conditions (Fig. 3 and Supplementary Fig. 2). Increasing salt concentration decreased the lytic activity of all six endolysins. However, phiCD38-2_gp23_EAD

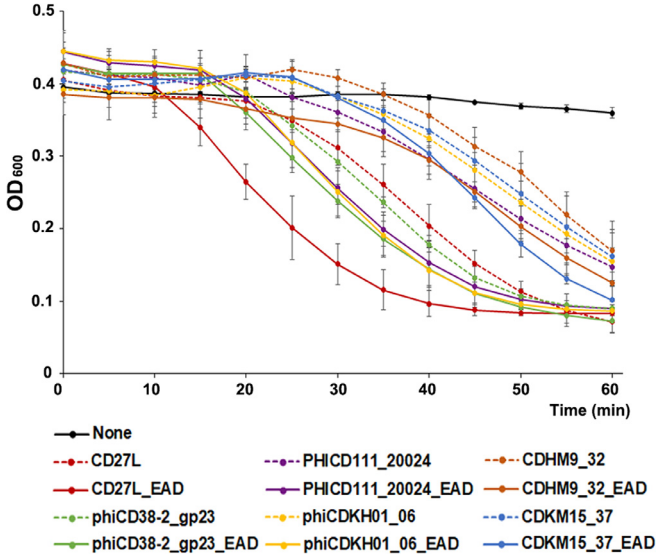

Figure 2. Comparison of lytic activity between truncated and full-length endolysins. *Clostridioides difficile* ATCC 9689 cells in the logarithmic growth phase were treated with six full-length endolysins (dotted line) and their truncated forms (solid line) at 0.16 μM in 20 mM Tris-HCl (pH 7.5) buffer. Turbidity reduction was measured at optical density at 600 nm for 1 h. "None" indicates no endolysin was added.

and PHICD111_20024_EAD maintained high enzymatic activity at concentrations over 100 mM. Under varied pH values from 5.5 to 9.5, three endolysins, i.e., CD27L_EAD, phiCD38-2_gp23_EAD, and PHICD111_20024_EAD, maintained stable activity. CD27L_EAD showed high activity, even at pH 5.5. When the endolysins were pre-incubated under different temperatures from 4 to 65°C for 30 min, CD27L_EAD and PHICD111_20024_EAD exhibited higher activities than the others up to 55°C. Intriguingly, CD27L_EAD showed comparable lytic activity even after 65°C treatment.

To evaluate the enzymatic specificity, 26 bacterial species, including 18 gram-positive and eight gram-negative species, were treated with six recombinant endolysins. Six *C. difficile* strains were susceptible to the six endolysins in the turbidity reduction (Table 1 and Supplementary Fig. 3A) and viability (Supplementary Fig. 3B) assays. Although the majority of other gram-positive bac-

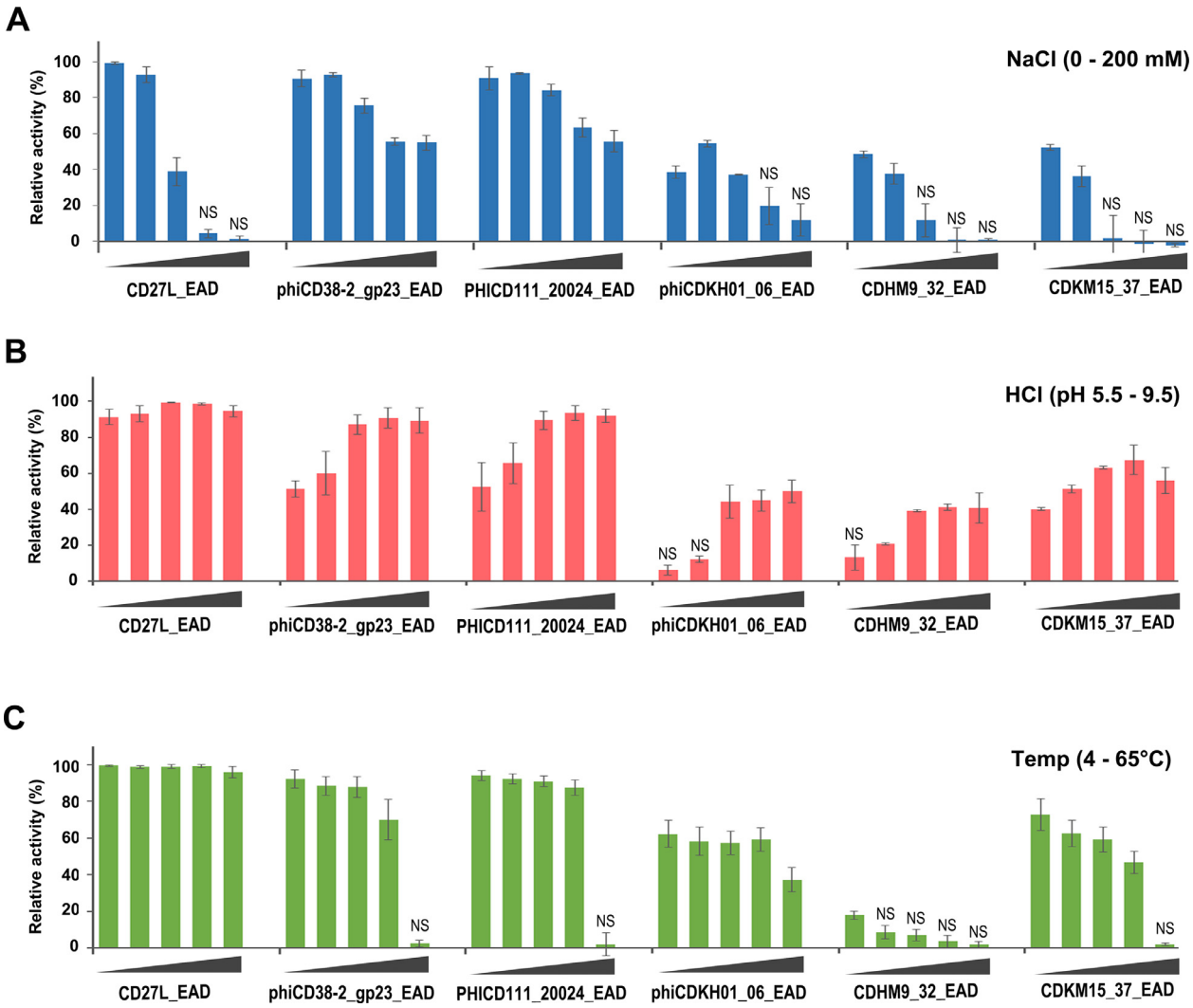

Figure 3. Lytic activity of six recombinant endolysins in various salt, pH, and temperature conditions. Bacteriolytic activities of the six recombinant endolysins against *Clostridioides difficile* ATCC 9689 were compared under various salt (a), pH (b), and temperature (c) conditions. All endolysins were used at 0.16 μM at 37°C in 20 mM Tris-HCl. Salt concentration and pH were adjusted using NaCl (0, 50, 100, 150, and 200 mM) and HCl (pH 5.5, 6.5, 7.5, 8.5, and 9.5), respectively. To test thermostability, endolysins were pre-treated at different temperatures (4, 37, 45, 55, and 65°C) for 30 min and applied to *C. difficile* at 37°C. Turbidity reduction was measured at optical density at 600 nm (OD_{600}) at 30 min and lytic activity was assessed as follows: $\{(OD_{600[untreated]} - OD_{600[treated]}) / (OD_{600[untreated]})\} \times 100\%$. A value with an insignificant difference from the level of untreated control is denoted by NS ($P < 0.05$).

teria were not vulnerable to the six endolysins, strains of *Bacillus* spp., *Clostridium* spp., and *Listeria* spp. were susceptible. Six full-length endolysins also exhibited moderate to substantial lytic activity against strains of these genera (Supplementary Fig. 4). These results suggest that N-terminal EADs possess considerable potential for determining the substrate specificity of the six endolysins. Fourteen gram-negative bacterial strains were not susceptible to any of the endolysins.

3.3. Synergistic killing activity of CD27L_EAD with vancomycin

The bacterial spectra of the six truncated endolysins were similar; however, CD27L_EAD showed high and stable activity against *C. difficile* under various conditions. Therefore, CD27L_EAD was selected to inactivate *C. difficile*. CD27L_EAD had a MIC value at 640 μM against *C. difficile* (Fig. 5A). To promote its inhibitory effect, vancomycin was added in combination, and the FIC indices were determined (Fig. 4A). In the presence of vancomycin at 1 μg/mL, CD27L_EAD ranging from 40 to 160 μM exerted synergistic inhibitory effects [23]. Human fecal microbiota was mixed with *C. difficile* cells at a 100:1 ratio and cultivated in BHI medium broth supplemented with CD27L_EAD (160 μM), vancomycin (1 μg/mL), or both. The pH values and salt concentrations were measured during the cultivation (Supplementary Fig. 5). The number of *C. difficile* cells was determined by qPCR (Fig. 4B). *C. difficile* showed a 10.7-fold increase in viability after 6 h in the absence of CD27L_EAD and vancomycin. However, CD27L-EAD treatment decreased the number by approximately 9.3-fold after 6 h, and its combination with vancomycin resulted in an approximately 47-fold reduction after 6 h. Interestingly, vancomycin alone did not attenuate *C. difficile*.

3.4. Metagenomic analysis on CD27L_EAD-treated gut microbiota

To examine the possibility of gut microbiota disturbance by CD27L_EAD, metagenomics was conducted on human fecal microbiota cultivated with CD27L_EAD at 640 μM (Supplementary Data 1). Taxonomic classification based on species-level operational taxonomic units (OTUs) revealed that relative abundance profiles were indistinguishable between CD27L_EAD–treated and untreated specimen (Fig. 5A). Evaluation of β-diversity based on PCoA using the Bray–Curtis dissimilarity index showed that the culture condition itself caused alterations in the microbial composition and

Table 1
Lytic activity of six recombinant endolysins against other bacterial species.

Endolysin bacterial strain		CD27L_EAD	phiCD38-2_gp23_EAD	PHICD111_20024_EAD	phiCDKH01_06_EAD	CDHM9_32_EAD	CDKM15_37_EAD
Gram (+)	*Clostridioides difficile* ATCC 9689	++	++	++	++	++	++
	C. difficile ATCC 43599	++	++	++	++	++	++
	C. difficile ATCC 43600	++	++	++	++	++	++
	C. difficile NCCP 11080	++	++	++	++	++	++
	C. difficile NCCP 11820	++	++	++	++	++	++
	C. difficile NCCP 11840	++	++	++	++	++	++
	Bacillus cereus ATCC 14579	-	-	-	-	-	-
	B. cereus NCCP 10623	-	-	-	-	-	-
	B. cereus NCCP 10624	+	+	+	+	-	+
	Bacillus subtilis ATCC 23857	+	+	+	+	+	+
	B. subtilis SRCM103551	+	+	+	+	+	+
	Bifidobacterium adolescentis ATCC 15703	-	-	-	-	-	-
	Clostridium perfringens ATCC 13124	-	-	-	-	-	-
	C. perfringens NCCP 15911	-	-	-	+	-	-
	Clostridium sporogenes KCTC 5654	+	++	++	++	++	+
	Clostridium tertium KCTC 5897	+	+	+	+	+	+
	Enterococcus faecalis ATCC 29212	-	-	-	-	-	-
	E. faecalis CCARM 5537	-	-	-	-	-	-
	Enterococcus faecium CCARM 5202	-	-	-	-	-	-
	Lactobacillus gasseri ATCC 33323	-	-	-	-	-	-
	Lactobacillus paracasei KCTC 3510	-	-	-	-	-	-
	Lactobacillus plantarum ATCC 8014	-	-	-	-	-	-
	Listeria innocua KCTC 3586	+	+	+	+	+	-
	Listeria ivanovii KCTC 3444	+	++	+	+	+	-
	Listeria monocytogenes KCTC 3569	+	++	+	+	+	-
	Staphylococcus aureus ATCC 29213	-	-	-	-	-	-
	S. aureus CCARM 3689	-	-	-	-	-	-
	S. aureus KCTC 1928	-	-	-	-	-	-
	Staphylococcus epidermidis ATCC35983	-	-	-	-	-	-
	Streptococcus pyogenes ATCC 19615	-	-	-	-	-	-
Gram (-)	*Acinetobacter baumannii* ATCC 19606	-	-	-	-	-	-
	A. baumannii F-750	-	-	-	-	-	-
	Cronobacter sakazakii ATCC 29544	-	-	-	-	-	-
	Escherichia coli ESBL HID5005	-	-	-	-	-	-
	E. coli K03-Bact-08-037	-	-	-	-	-	-
	E. coli MG1655 ATCC 700926	-	-	-	-	-	-
	Klebsiella pneumoniae ATCC 700721	-	-	-	-	-	-
	K. pneumoniae 1366019	-	-	-	-	-	-
	K. pneumoniae 140522-4113	-	-	-	-	-	-
	Pseudomonas aeruginosa ATCC 27853	-	-	-	-	-	-
	Salmonella IVK B01009	-	-	-	-	-	-
	Salmonella Typhimurium ATCC 14028	-	-	-	-	-	-
	Vibrio cholerae ATCC 14033	-	-	-	-	-	-
	Yersinia enterocolitica ATCC 55075	-	-	-	-	-	-

Lytic activity was calculated as follows: $\{(OD_{600[untreated]} - OD_{600[treated]})/(OD_{600[untreated]})\} \times 100\%$.
++, strong lytic activity ≥ 80%; +, moderate lytic activity < 80%; -, no lytic activity < 20%.

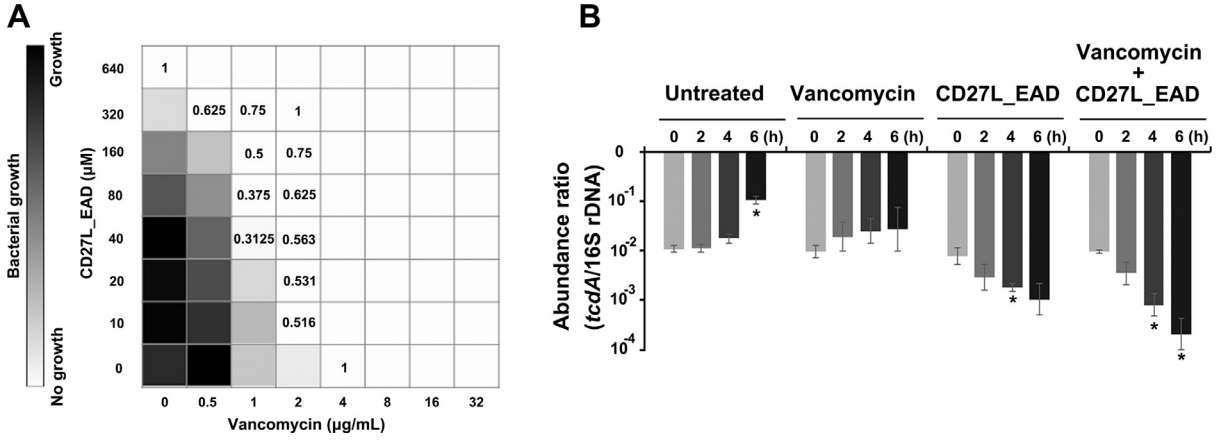

Figure 4. Synergistic lytic activity of CD27L_EAD with vancomycin. (a) Potency of combination of CD27L_EAD and vancomycin was determined using a checkerboard assay. *Clostridioides difficile* cells were treated with different concentrations of two agents in combination in brain heart infusion (BHI) broth. Bacterial growth was measured at optical density at 600 nm after 24 h and presented in a heatmap. Minimal inhibitory concentrations (MICs) were determined at 640 μM (CD27L_EAD) and 4 μg/mL (vancomycin), respectively. Fractional inhibitory concentration (FIC) index was calculated using MICs as described in Materials and Methods. FIC indices with synergistic (≤0.5) or additive (0.5–1) effects are indicated. (b) Relative abundance of *C. difficile* was quantified using qPCR after CD27L_EAD and vancomycin treatments. Fecal microbiota and *C. difficile* cells were mixed at a 100:1 ratio and cultivated in BHI broth supplemented with CD27L_EAD (160 μM), vancomycin (1 μg/mL), or both. Primers specific to *tcdA* were used to estimate number of *C. difficile*, whereas 341F/R806 primers specific to 16S rDNA were used to quantify whole bacterial genome. Ct values of *tcdA* were normalized to those of 16S rDNA and relative abundance is plotted. A significant difference from 0 h in each treatment is denoted by an asterisk ($P < 0.05$).

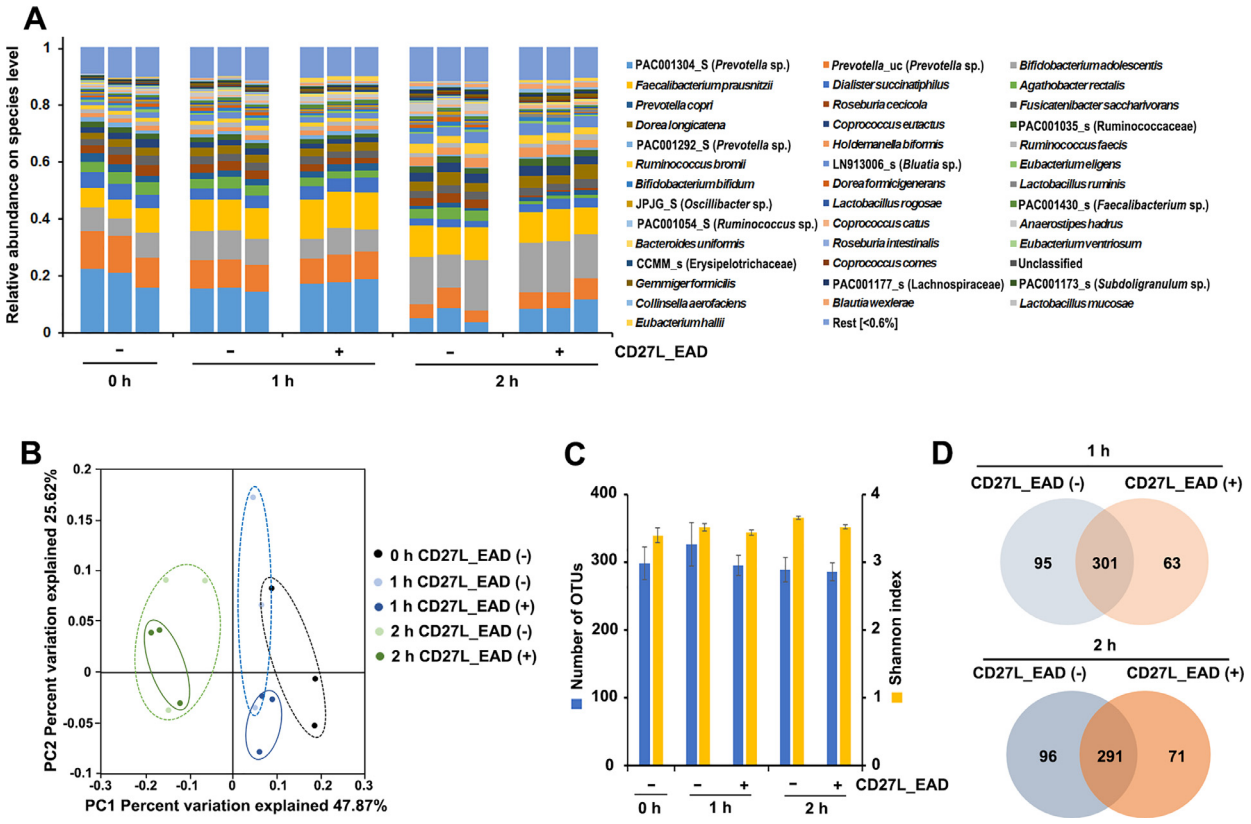

Figure 5. Metagenomics on gut microbiota after CD27L_EAD treatment. Gut microbiota (approximately 10^7 CFU/mL) harvested from 32-year-old healthy female was treated with CD27L_EAD at 640 μM and total genomic DNAs were analyzed using 16S rRNA sequencing. (a) Relative abundance of bacterial reads was classified at species level. (b) Overall community composition and structure were compared by β-diversity analysis using Bray–Curtis dissimilarity index. (c) α-diversity analysis was conducted using total number of operational taxonomic units (OTUs) and Shannon diversity index. (d) Venn diagrams showing overlapping bacterial species (OTUs) between treatments.

structure, but the microbial samples treated or untreated with CD27L_EAD appeared to cluster together, indicating the similarity between their microbiota compositions (Fig. 5B). α-diversity analysis using Shannon and OTU count indices suggested that bacterial population diversity was not impaired by CD27L-EAD treatment (Fig. 5C). The total number of identified OTUs from the CD27L_EAD–treated and untreated specimens was 362 and 387, respectively, sharing 291 OTUs between the two groups at 2 h (Fig. 5D). CD27L_EAD had no cytotoxicity against Caco-2 cells for 24 h (Supplementary Fig. 6).

3.5. *Metagenomic analysis on* C. difficile*-contaminated gut microbiota after CD27L_EAD treatment*

CD27L_EAD alone or in combination with vancomycin was applied to an *in vitro* CDI model comprising fecal microbiota and *C. difficile* at a 100:1 ratio, and changes in the bacterial community structure were investigated using 16S rRNA gene sequencing (Supplementary Data 2). Relative abundance of phylum-level OTU taxonomic classification was not significantly altered by CD27L_EAD, vancomycin, or the combination of the two (Supplementary Fig. 7). In the species-level analysis, changes by the cultivation condition were observed, and some species, such as *Mitsuokella multacida* lowered their abundance even after 2 h of cultivation. However, antimicrobial agents (CD27L_EAD, vancomycin, or both) did not appear to cause significant alterations in the structure or diversity of the *C. difficile*-contaminated fecal microbiota (Fig. 6A). The abundance of *C. difficile* was plotted separately (Fig. 6B). In accordance with the qPCR results (Fig. 4B), CD27L_EAD alone successfully decreased the abundance of *C. difficile* and co-administration with vancomycin resulted in a remarkable attenuation even at 2 h. Their inhibitory effects were substantial for at least 6 h. α-diversity analysis indicated that species richness and evenness were not damaged by CD27L_EAD or vancomycin (Supplementary Fig. 8A). The numbers of OTUs that were unique to each treatment or shared across treatments are depicted using Venn diagrams (Supplementary Fig. 8B). PCoA plotting demonstrated that some treatments, such as 2 h or 6 h CD27L_EAD, showed a higher spread than the other treatments, indicating higher inter-sample variability in these treatments (Fig. 6C). However, at each time point, the bacterial specimens appeared to cluster together, regardless of the added antimicrobial agents. These results suggest that the community structure was not significantly disturbed by CD27L_EAD or vancomycin treatments, either individually or in combination.

The relative abundance of each species was computed to identify the bacterial species whose abundance was substantially influenced by CD27L_EAD (Fig. 7). As observed in the abundance profiles (Fig. 6A), the *in vitro* cultivation condition itself (Untreated, Fig. 7) altered the abundance of some species, including a decreased abundance of *M. multacida* and *Clostridium butyricum* and an increased abundance of *Eubacterium hallii, Blautia wexlerae*, and *Bacteroides dorei*. To offset the effects of cultivation conditions, untreated controls at 2, 4, and 6 h were used to normalize the changes in each treatment. Intriguingly, CD27L_EAD significantly decreased the abundance of bacterial species belonging to the Lachnospiraceae family, including *Dorea formicigenerans, Agathobacter rectalis, Eubacterium ventriosum, Roseburia inulinivorans, Roseburia intestinalis*, and *Eubacterium ramulus*, although some Lachnospiraceae species flourished after CD27L_EAD treatment. Lachnospiraceae was the second most abundant family in

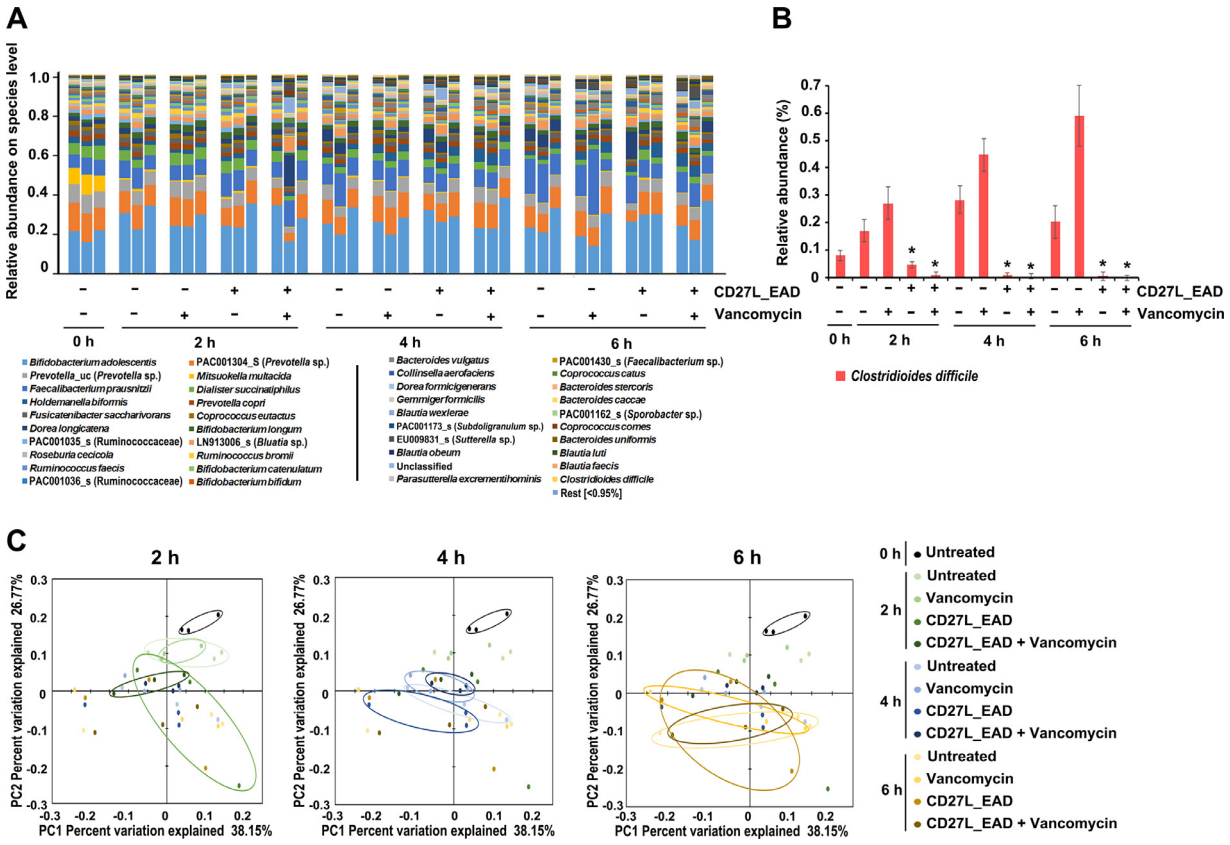

Figure 6. Metagenomics on *Clostridioides difficile*-contaminated gut microbiota after CD27L_EAD treatment. Brain heart infusion broth was inoculated with *Clostridioides difficile* (approximately 10^5 CFU/mL) and gut microbiota (approximately 10^7 CFU/mL). Vancomycin (1 μg/mL) and CD27L_EAD (160 μM) were added individually or in combination. Total genomic DNA was harvested at 0, 2, 4, and 6 h, and subjected to 16S rRNA sequencing. (a) Relative abundance of bacterial reads was classified at species level. (b) Relative abundance of *C. difficile* was determined based on number of reads. A significant difference from the level of untreated control at each time point is denoted by an asterisk ($P < 0.05$). (c) β-diversity analysis using Bray–Curtis algorithm was applied to estimate compositional dissimilarity between treatments. Samples at same time points were grouped and colored separately.

the intact fecal microbiota (Supplementary Fig. 9). Notably, species belonging to the family Peptostreptococcaceae, except for *C. difficile*, were not vulnerable to CD27L_EAD treatment.

4. Discussion

Antibiotics such as vancomycin and fidaxomicin are currently the first-line treatments for CDI. However, antibiotics, as double-edged swords, have the potential to aggravate CDI through gut microbiota dysbiosis and re-colonization with antibiotic-resistant *C. difficile* [6,7]. As an alternative to antibiotics, endolysins are generally species-specific and are less likely to disrupt the commensal gut microbiota. Furthermore, the emergence of resistant bacteria is practically impossible [15]. In the present study, we identified six endolysins with lytic activity against six *C. difficile* strains. A BLAST search predicted that all six endolysins possessed catalytic domains with *N*-acetylmuramoyl-L-alanine amidase activity. As observed in a previous study [19,20], truncated derivatives containing only the catalytic domains showed higher lytic activity than their cognate full-length forms and retained substrate specificity. Of the six derivatives, CD27L_EAD exhibited the highest lytic activity against the tested *C. difficile* strains. However, truncated endolysins also showed slightly increased lytic activity against less closely related species, i.e., *Bacillus cereus, Bacillus subtilis, Clostridium sporogenes, Clostridium tertium, Listeria innocua, Listeria monocytogenes*, and *Listeria ivanovii*. These vulnerable species as well as *C. difficile* have A1γ-type peptidoglycan architecture, where the ε-amino group of the m-Dap residue at position 3 of one peptide forms a peptide bond directly with the carboxyl group of D-Ala at position 4 of an adjacent peptide subunit [31]. However, *Lactobacillus plantarum*, whose peptidoglycan type is also A1γ, was resistant to the truncated endolysins. Notably, the N-terminal catalytic domain mainly determined substrate specificity, and deletion of the C-terminal cell wall-binding moiety did not alter the host range of the full-length endolysin. The dual role of the N-terminus exerting the lytic activity and determining the host range was previously reported in CD27L produced by Phage ΦCD27 [19]. Phage ΦCD27, a member of the Myoviridae, was first identified using mitomycin C induction of *C. difficile* NCTC 12727 [32]. Mayer et al. also observed that a truncated variant of CD27L ($CD27L_{1\text{-}179}$) containing only an N-terminal EAD caused more rapid lysis than full-length CD27L, but retained the same spectrum [19].

When CD27L_EAD was applied to human gut microbiota inoculated with *C. difficile*, most of the commensal microbiota (71.5%; 316 of 442 species) was conserved after 6 h of endolysin treatment. Bacterial species whose abundance was attenuated more than 4-fold by CD27L_EAD were predicted to belong to the family Lachnospiraceae. Interestingly, bacterial species of the family Clostridiaceae, including *Clostridium leptum, Clostridium innocuum*, and *Clostridium butyricum*, did not decrease in abundance after CD27L_EAD treatment, indicating a distinct cell wall structure of *C. difficile* from that of *Clostridium* spp. Lachnospiraceae and Clostridiaceae are among the most abundant taxa in the human gut microbiota and produce short-chain fatty acids (SCFAs) such as butyrate and acetate via the fermentation of diverse dietary polysaccharides [33,34]. Due to the functional correlation of SCFAs with micro-

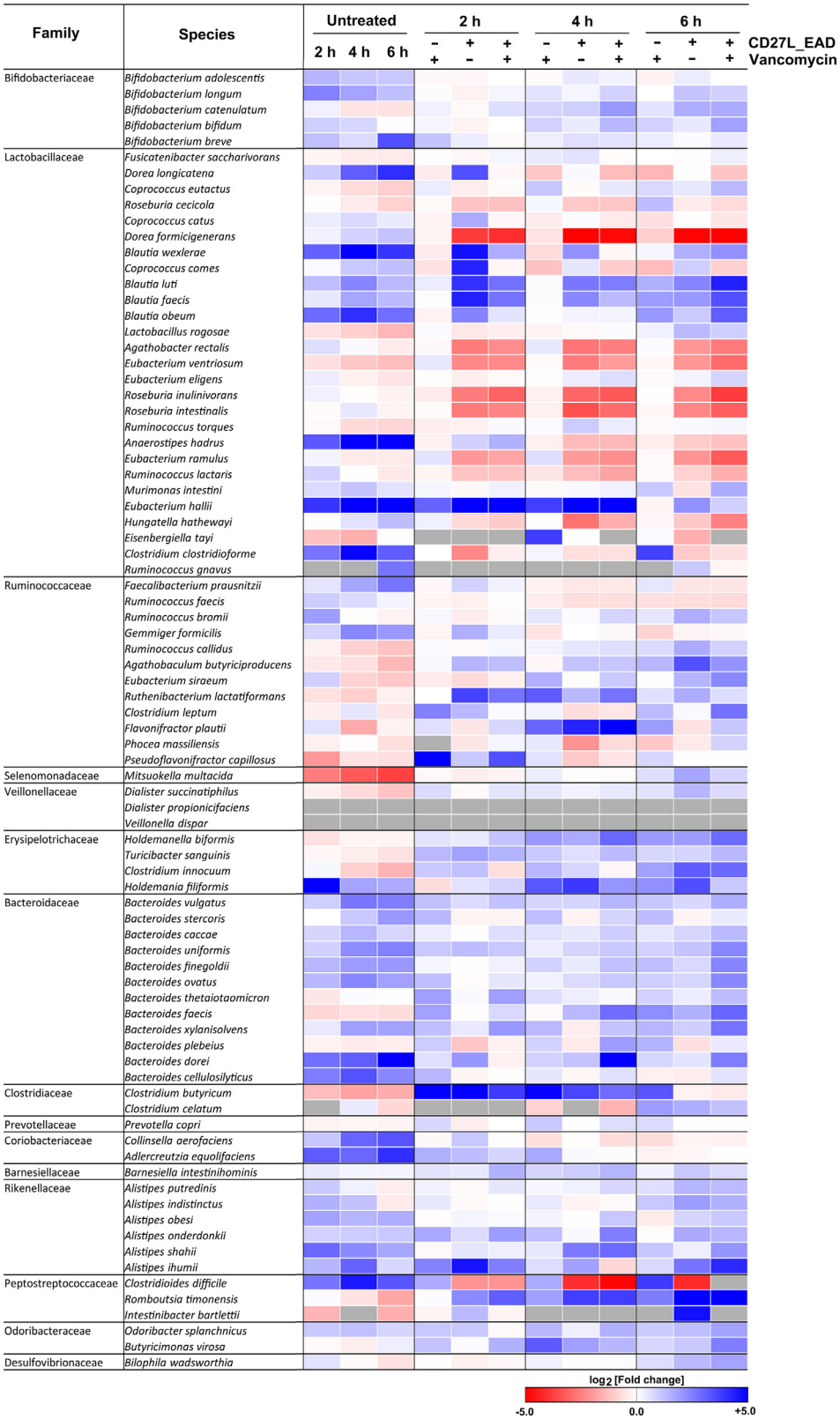

Figure 7. Bacterial species with altered abundance after CD27L_EAD and vancomycin treatments. Gut microbiota (approximately 10^7 CFU/mL) and *Clostridioides difficile* (approximately 10^5 CFU/mL) were mixed and cultivated with vancomycin (1 μg/mL) and CD27L_EAD (160 μM), individually or in combination. Total genomic DNA was harvested at 0, 2, 4, and 6 h, and subjected to 16S rRNA sequencing. Bacterial species belonging to top 15 family groups were sorted and their relative abundances were computed using number of reads. For untreated samples, abundance at 0 h was used to normalize levels at different time points (2, 4, and 6 h). When treated with CD27L_EAD or vancomycin, abundance of untreated samples at each time point was used as a control. Abundance ratio is expressed in $\log_2$[test/control]: + value (blue), increase; - value (red), decrease; 0 (white), no change. Grey indicates "not determined."

bial and host metabolism, members of these families have been associated with human diseases, such as colon cancer, obesity, and diabetes [34–36]. Notably, not all species belonging to Lachnospiraceae decreased in abundance in response to CD27L_EAD treatment; however, some members, including *B. wexlerae, Blautia luti, Blautia faecis*, and *Eubacterium hallii*, increased in abundance after endolysin treatment. *Blautia* spp. and *E. hallii* are primarily engaged in SCFA production in the intestinal tract [37,38].

Intriguingly, vancomycin alone failed to mitigate *C. difficile* proliferation in the gut microbiota model while dampening the abundance of members of Lachnospiraceae, including *Dorea longicatena, Coprococcus comes*, and *Dorea formicigenerans*. However, the combination of CD27L_EAD and vancomycin exerted better therapeutic effects than the single CD27L_EAD treatments, most likely because of the different modes of action of the two antimicrobial agents. Debranching of short peptide chains from the glycan backbone, which is attributable to CD27L_EAD, may enhance the accessibility of vancomycin to the D-Ala-D-Ala peptide motif of the peptidoglycan precursor. The synergistic therapeutic effect by the co-administration with vancomycin has been demonstrated in other amidase endolysins as well [20].

Three recombinant endolysins, CD27L_EAD, phiCD38-2_gp23_EAD, and PHICD111_20024_EAD, showed high lytic activity under varied pH conditions (pH 5.5–9.5) but CD27L_EAD showed the highest lytic activity even at pH 5.5. The human gastrointestinal tract is constantly exposed to ingested foods and the intraluminal pH is dynamically changed along the gastrointestinal tract: a strong acidity at pH 1.0–2.0 in the stomach, increases to an average pH 6.1 in the duodenum, gradually increases to pH 7.5 in the terminal ileum, temporally decreases to pH 6.0 near the cecum, and gradually increases to approximately pH 7.0 near the exit of the rectum [39]. The salinity of the gut contents is also influenced by many factors, such as age, diet, water intake, and luminal structure, and fluctuates at approximately 1%, equivalent to 170 mM NaCl [40,41]. Two truncated endolysins, phiCD38-2_gp23_EAD and PHICD111_20024_EAD, exhibited stable and high lytic activity even at 200 mM NaCl, in contrast to CD27L_EAD. In view of the complex environment of the human gut, there are still several challenges to overcome, such as more sophisticated substrate specificity and more stable enzymatic activity. A critical challenge of using endolysins in a clinical setting is attributable to the proteinaceous nature of endolysins. Oral administration is generally not suitable because of stomach acid and multiple proteases, which render proteinaceous enzymes inactive. To detour this obstacle, Wang et al. attempted rectal administration of *C. difficile* endolysin PlyCD1-174 by enema in a mouse model but failed to reproduce the therapeutic effect [20]. In order to circumvent these challenges, diverse delivery systems have been explored, including nanoparticle-based encapsulation [42] and transgenic probiotics [43] as a vehicle. Mayer et al. manipulated *Lactococcus lactis* MG1363 to produce CD27L and showed the lysate of engineered *Lactococcus* strain exhibited substantial lytic activity against *C. difficile* [32]. A robust drug delivery system and an appropriate animal platform should be developed in parallel to accelerate the application of endolysins to a clinical setting.

Antibiotics with broad-spectrum are prone to result in gut microbiota perturbations and recurrent CDI. CD27L and its truncated variant $CD27L_{1-179}$ have been explored as a promising therapeutic agent against CDI and their selective lytic activities were evaluated *in vitro* using many different bacterial species including other clostridial species [19,32]. One of the highlights of this study was that the possibility of gut dysbiosis by CD27L_EAD was examined using human gut microbiota. Structural profiling of the gut microbiota revealed that CD27L_EAD progressively decreased the abundance of *C. difficile* over time and was less likely to cause collateral damage to the commensal bacterial community. Furthermore, its combination with vancomycin almost completely eradicated *C. difficile* after 6 h of treatment. With regard to mitigating CDI without compromising the natural gut microbiota, this study sheds light on endolysin as a potential therapeutic agent.

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Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.ijantimicag.2024.107222.

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